University of Alberta

Mate choice and sperm allocation in male goldfish (*Carassius auratus*): Effect of female size and competitors

by

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ABSTRACT

In many fishes, female fecundity increases with size, and males exhibit preference for larger females through differential courtship, spawning, and/or sperm release. I investigated the effect of female size and male competitors on mate choice, sperm allocation and endocrine response in goldfish, a fish with a scramble competition mating system. Mate choice was assessed by allowing one male to interact simultaneously with two sexually receptive females, in one of three treatment groups: *smaller* (male smaller than both females), *intermediate* (male between the female weights) and *larger* (male larger than both females). Intermediate and larger males preferentially courted and spawned with large females but smaller males did not. Sperm allocation and endocrine response (serum luteinizing hormone, testosterone) were not influenced by female size or the presence of competitors. My results suggest that sperm allocation in male goldfish is determined by behavioral (partner choice) rather than by physiological (differential sperm production) mechanisms.

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1. INTRODUCTION

Sperm competition occurs when sperm from different males compete to fertilize a female's eggs (Parker, 1970) and has been well studied in a number of diverse animal groups (Gage, 1994; Hosken, 1997; Macias-Garcia and Saborio, 2004; Schulte-Hostedde and Millar, 2004; Stockley and Preston, 2004; Tuttle and Pruett-Jones, 2004: reviewed in Birkhead and Møller, 1998). Although extensive research in this area has elucidated some of the factors influencing sperm competition, our understanding is incomplete and complicated by the variety of mating systems.

In internally fertilizing species, males should partition reproductive investment based on the *risk* of sperm competition (Wedell et al., 2002). Thus, when the probability is high that a female will mate again before all eggs are fertilized, males should increase sperm investment (Wedell et al., 2002) as has been shown in birds (Nicholls et al., 2001; Pizzari et al., 2003) and mammals (delBarco-Trillo and Ferkin, 2004). Alternatively, males may attempt to reduce sperm competition by sperm displacement, mating plugs, or guarding behaviors, as occurs in many insect species, where fertilization is internal (Parker, 1970). In contrast, males of externally fertilizing species should partition reproductive investment based on the *intensity* of sperm competition, which may be assessed directly by observing the number and nature of competitors (Ball and Parker, 1997). Strategies of sperm competition may include modulating sperm quantity and sperm quality or investing energy into reproductively monopolizing behaviors (Taborsky, 1998).

1.1. Sperm Competition in Externally Fertilizing Fishes

Parker (1990a) proposed that in externally fertilizing fishes where gametes are synchronously released into the surrounding environment, sperm competition proceeds by a *fair raffle*. In a fair raffle, males increase the proportion of eggs fertilized by increasing sperm release (Parker, 1990a). However, because the cost of producing sperm is non-trivial (Dewsbury, 1982), the competitor who invests all his energy into reproduction will have nothing to invest in growth and consequently will decrease his chances of survival and future reproductive success (Parker, 1990a). As a result, males of iteroparous species should increase sperm production based on an evolutionary stable strategy (ESS; Maynard-Smith, 1982) that balances the intensity of sperm competition and the cost of production of ejaculates. In contrast, males of semelparous species, should invest in sperm production at the expense of growth or future reproduction. Furthermore, in the hypothetical situation where all males have equal resources to invest in sperm production, it is expected that there will necessarily be a negative correlation between sperm quantity and sperm quality. Hence, the sperm of high output males is devalued due to its assumed inferior quality and reduced ability to fertilize eggs (Parker, 1990a). In this case, sperm competition proceeds by a *loaded raffle*. Males should produce sperm based on an ESS that optimizes sperm quantity and quality.

Sperm quality is defined by the capacity of the sperm to fertilize an egg (Rurangwa et al., 2004). Consequently, any factor relating to sperm and correlating with fertilization capacity may act as an indicator of sperm quality (Rurangwa et al., 2004). Possible indicators of sperm quality include 1) physical factors (motility, morphology), 2) energy reserves (concentration of adenosine triphosphate), 3) enzymatic activity (malate

dehydrogenase, aspartate aminotransferase) and 4) characteristics of the seminal plasma (osmolarity, chemical composition) (Aas et al., 1991; Lahnsteiner et al., 1998: reviewed in Rurangwa et al., 2004).

The sperm of most teleosts, including the common carp (*Cyprinus carpio*), lack an acrosome and remain immotile in the genital tract and semen until activation by release into the water (Billard et al., 1995). The duration of motility in the common carp is relatively short, only 30-40 seconds (Billard et al., 1995). In contrast, the duration of motility in the Atlantic cod (*Gadus morhua*) may last for 10 minutes (Trippel and Neilson, 1992). With some exceptions (Aas et al., 1991; Trippel and Neilson, 1992; Hoysak and Liley, 2001), sperm motility (duration or proportion of motile sperm) is positively correlated with fertilization capacity in some externally fertilizing fishes (Moccia and Munkittrick, 1987; Levanduski and Cloud, 1988; Fauvel et al., 1999; Alavi and Cosson, 2005).

Although the quality-quantity trade-off is an inherent aspect of the loaded raffle, experimental results are unclear. A comparative study on a wide variety of fish species from 26 families indicates that males from species with mating systems involving intense sperm competition have shorter sperm (defined as the total length of the sperm) (Stockley et al., 1997). Although this finding might appear to support the idea of a quantity-quality trade-off in sperm competition (Stockley et al., 1997), shorter sperm do not appear to be of lower quality, as sperm length is negatively correlated with duration of motility (Stockley et al., 1997). Similarly, in Atlantic salmon (*Salmo salar*), sperm length does not correlate with sperm velocity (Gage et al., 2002) or sperm motility (duration of motility and proportion of motile sperm) (Gage et al., 1998). Assuming that the

measures above are positively correlated with fertility, it appears that larger sperm are not of better quality. Further, *in vitro* experiments that attempted to simulate natural fertilization conditions and that used microsatellite DNA fingerprinting to assess paternity suggested that total sperm length is not associated with fertilization success and that the primary factor associated with fertilization success is relative sperm velocity (Gage et al., 2004).

Additional data that are inconsistent with the quantity/quality trade-off are found in cichlids. In polygamous cichlids, where the risk of sperm competition is high, males produce larger sperm (Balshine et al., 2001), as do males in some polyandrous, internally fertilizing primates and rodents (Gomendio and Roldan, 1991) and in nematodes (LaMunyon and Ward, 1999). In the hermaphroditic nematode *Caenorhadbitis elegans*, large sperm appear to be of superior quality because of increased sperm velocity and their occupation of preferential areas in the reproductive tract (LaMunyon and Ward, 1998).

Behavioral competition may also influence sperm competition among males that use the same mating strategy. For example, in the situation where two males producing the same quantity and quality of sperm are in direct competition to fertilize the eggs of a single female, the male that releases his sperm closest to the eggs should have a greater probability of fertilizing them. In species such as the goldfish (*Carassius auratus*), where the mating system involves scramble competition (Anderson, 1994; Spritzer et al., 2005), it is expected that males will behaviorally compete for a position closest to the female.

In some species of fish, males engage in alternative mating strategies as a means of sperm competition. *Bourgeois* males, those able to defend a female or resource, invest energy into guarding behavior, whereas *parasitic* males, which usually are small and

precocious, attempt to gain brief access to females for sneak matings (Taborsky, 1998). Where the occurrence of sneak matings is low, it is predicted that large males will invest relatively more energy into mate guarding than sperm production (Parker, 1990b). Parasitic males should increase sperm production because, in the event that they are successful in their clandestine mating, their large quantities of sperm will fertilize a greater proportion of the eggs than the sperm of the bourgeois male (Parker, 1990b). In the bluegill sunfish (*Lepomis macrochirus*), a species with bourgeois and parasitic males, parasitic males have higher sperm densities (Neff et al., 2003) and fertilize a greater proportion of the eggs (in the specific spawning events in which they participate) than bourgeois males (Fu et al., 2001). As the probability of sneak matings increases, theory suggests that large males should invest proportionally more into sperm production (Parker, 1990b); however, research on the Mediterranean wrasse (*Symphodus ocellatus*) suggests that this is not the case. The territorial male engages in more chasing behavior as the number of parasitic males increases (Alonzo and Warner, 2000), providing evidence that bourgeois males invest more energy into behaviors that reduce sperm competition.

1.2. Sperm Allocation

Sperm allocation is a reproductive strategy that attempts to maximize reproductive success over the entire mating period. In the situation where a male is unable to produce sufficient number of ejaculates to fertilize all the eggs he may encounter, a male must balance his investment in reproduction and adopt a mating strategy that allocates sperm in such a way as to maximize reproductive success. This

may mean producing a greater number of sperm, producing a better quality of sperm (increased motility, duration of motility), or releasing different numbers of sperm in response to the perceived number of competitors. Because sperm are small and lack cytoplasm, the cost of an individual spermatozoan is trivial when compared to that of an egg. However, because males usually release ejaculates containing millions of sperm, the cost of producing an ejaculate may be a limitation to fertility (Dewsbury, 1982). In situations where males engage in more than one ejaculatory event, selection should favor those males best able to allocate their limited sperm resource to achieve the greatest number of fertilizations.

Although sperm allocation has been studied in a number of fishes (bluehead wrasse, *Thalassoma bifasciatum*; Shapiro et al., 1994: Atlantic salmon, *Salmo salar*; Gage et al., 1995: bucktooth parrotfish, *Sparisoma radians*; Marconato and Shapiro, 1996: rainbow darter, *Etheostoma caeruleum*; Fuller, 1998a: Mediterranean wrasse, *Symphodus ocellatus*; Alonzo and Warner, 2000: black goby, *Gobius niger*; Pilastro et al., 2002: grass goby, *Zosterisessor ophiocephalus*; Pilastro et al., 2002), these species all employ mating systems in which females mate with a bourgeois male. However, no study has investigated sperm allocation in a promiscuous species with a scramble competition mating system such as goldfish.

The goldfish *(Carassius auratus)* is a temperate zone fish that is externally fertilizing and lives in bisexual groups. Based on Balon's scheme for categorizing the reproductive styles of fishes, the goldfish is a non-guarding, open-substrate egg scatterer that is an obligatory plant spawner (i.e. *phytophil*; Balon, 1984). Females can ovulate several times over a spawning season, usually in late spring to early summer when water

temperature increases (Stacey and Sorensen, 2002; Stacey and Sorensen, 2006), and their parental care is restricted to the placement of the eggs in the vegetation. Males are promiscuous and do not guard females or territories, their only reproductive function being fertilization. Female goldfish release *hormonal pheromones* (i.e. released hormonal steroids, prostaglandins, and their metabolites) that signal their reproductive state, increase the quantity and quality in the sperm ducts, and induce male sexual behaviors (Kobayashi et al., 2002). Both the closely related crucian (*Carassius carassius*) and common carp (*Cyprinus carpio*), which also engage in intense sperm competition (Stockley et al., 1997), exhibit very similar mating and sex pheromone systems (Stacey et al., 1994; Olsén et al., 2006), and are considered to engage in intense sperm competition.

1.3. The Effect of Competitors on Sperm Allocation

In the goldfish mating system, sperm competition is expected to be chronic and variable in intensity. Over the mating season, a male must compete with unpredictable numbers of male rivals for an unpredictable number of females. Sperm competition is expected to proceed as predicted in externally fertilizing fishes that do not guard resources or mates (Parker et al., 1996, Ball and Parker, 1997). Based on theory, in the absence of a competitor, males should allocate minimal numbers of sperm to each reproductive event because even low numbers of sperm should ensure fertilization of the majority of eggs (Ball and Parker, 1997). In the presence of a single competitor, males should allocate maximal numbers of sperm because any added investment into sperm production will bias the probability of fertilizing eggs towards the male who produces

more sperm. As the number of competitors increases further, however, sperm release should decrease because the contribution of a single male will be insignificant when compared to the amount of sperm produced by the competitors (Ball and Parker, 1997). These predictions are not influenced by whether a male can judge the precise number of competitors, and all males are expected to conform to the same strategy as these behaviors constitute an ESS (Ball and Parker, 1997).

Although the predictions made by Ball and Parker (1997) regarding intensity of sperm competition and ejaculate size have yet to be tested in a non-guarding, externally fertilizing fish species, results from studies of guarding species are generally supportive. In the bitterling (*Rhodeus sericeus*), where males guard a freshwater bivalve into which females oviposit, bourgeois males increased ejaculation rate in the presence of a single competitor but decreased it with each additional competitor (Candolin and Reynolds, 2002). Further, in the rainbow darter, bourgeois males increase sperm release in the presence of competitors but do not vary the response based on the number of competitors (Fuller, 1998a). Similarly, in both the black and grass gobies, parasitic males increase sperm release in response to the visual presence of two additional parasitic males at the nest site and decrease sperm release in the presence of 3-5 parasitic males (Pilastro et al., 2002).

Evidence from the Mediterranean and bluehead wrasse species were inconsistent with the results above. In Mediterranean wrasse, bourgeois males do not alter the number of sperm released based on the number of parasitic males (Alonzo and Warner, 2000). In this species, males appear to have evolved behavioral rather than gametic competition. Similarly, in bluehead wrasse, parasitic males participating in group spawns (< 20 males)

released six times more sperm than males participating in paired spawns (Shapiro et al., 1994) suggesting that parasitic males do not decrease sperm release in the presence of numerous competitors.

1.4. The Effect of Female Body Size on Male Mate Choice and Sperm Allocation

In many internally fertilizing species (Bisazza et al., 1989; Honěk, 1993; Gage, 1998; Tammaru et al., 2002; Pizzari et al., 2003; Herdman et al., 2004; Marcías-Garcia and Saborío, 2004), female body size or sexual ornament size is positively correlated with fecundity. Also, in many species of externally fertilizing fishes, there is a positive relationship between female body size and fecundity (Fuller, 1998b; Michaletz, 1998; Jonsson and Jonsson, 1999; Loir et al., 2001; Wydoski, 2001; Pélabon et al., 2003; Sivakumaran et al., 2003). Large females represent the possibility for fertilization of a larger number of eggs and present males with the opportunity for greater reproductive success. Also, large females may produce eggs that are bigger, as well as more numerous (Kraak and Bakker, 1998; Dickerson, et al., 2002; Sivakumaran et al., 2003). Thus, males should favor spawning with large females and alter sperm production based on female body size.

Laboratory studies on the northern dusky salamander (*Desmognathus fuscus fuscus*; Marco et al., 1998), the western redback salamander (*Plethodon vehiculum*), the Dunn's salamander (*P. dunni*; Verrell, 1994) and the red-sided garter snake (*Thamnophis sirtalis parietalis*; Shine et al., 2003) provide some evidence that males prefer to court larger females. Further, studies on the internally fertilizing mosquitofish (*Gambusia holbrooki*) and guppy (*Poecilia reticulata*) indicate that males spend more time with the

large female and, as the size difference between females increases, so does the preference for the large female (Bisazza et al., 1989; Dosen and Montgomerie, 2004). In addition, sailfin mollie (*Poecilia latipinna*) males had a greater amount of sperm in their sperm ducts when placed with large females, suggesting that the males exposed to large females may release more sperm with these females (Aspbury and Gabor, 2004).

Similarly, behavioral studies of externally fertilizing fishes indicate that, when simultaneously presented with females of different sizes, males choose the large female (Sargent et al., 1986; Rosenqvist, 1990; Grant et al., 1995; Werner and Lotem, 2003; Wong and Jennions, 2003). In the Pacific blue-eye (*Pseudomugil signifer*), males preferred the large female when females were presented either simultaneously (Wong and Jennions, 2003) or sequentially (Wong et al., 2004), suggesting that the Pacific blue-eye has an absolute preference for large females. The preference for the large female was also observed in a natural setting where it was found that redlip blenny (*Ophioblennius atlanticus*) males preferentially accepted large females into their nests (Côte and Hunte, 1989).

Nevertheless, a behavioral preference for large females is not ubiquitous. In the two-spotted goby (*Gobiusculus flavescens*), male preference for the large female is weak (Pélabon et al., 2003). Males may use reproductive coloration as their primary basis of choice as they show a strong preference for colorful females (Amundsen and Forsgren, 2001). As well, in the orangethroat darter (*Etheostoma spectabile*; Pyron, 1996), sand goby (*Pomatoschistus minutus*; Kvarnemo and Forsgren, 2000) and zebrafish (*Danio rerio;* Pyron, 2003), preference for the large female is lacking. The absence of preference for the large female by male orangethroat darters and sand gobies may be due

to a male-biased sex ratio (Pyron, 1996; Kvarnemo and Forsgren, 2000). Additionally, in orangethroat darters there is little difference in the fecundity of females (Pyron, 1996). In each species, males are able to spawn with many females on the same day. Consequently, the pressure to choose the large female may be weak.

Males may use olfactory cues to differentiate between females of different size. In the red-sided garter snake, the percentage of unsaturated methyl ketones in the female sexual attractiveness pheromone is positively correlated with body size (LeMaster and Mason, 2002). Males directed more courtship displays towards large females (Shine et al., 2003) and towards filter paper blotted with skin lipids from large females (LeMaster and Mason, 2002; Shine et al., 2003). Also in salamanders (*Plethodon vehiculum* and *P. dunni*), males preferred the area of the tank containing the odor of the large female (Marco et al., 1998). This provides some evidence that males are able to distinguish female body size based on olfactory cues alone. However, this possibility has yet to be explored in fishes.

The effect of female body size on sperm allocation has been most comprehensively studied in the bluehead wrasse and bucktooth parrotfish. Both species are protogynous hermaphrodites in which males use alternate strategies to secure mating opportunities. Pair spawning usually occurs between females and bourgeois (i.e. *terminal phase*) males. Parasitic (i.e. *initial phase*) males may engage in sneak matings with a paired female or chase her and spawn in groups consisting of other parasitic males and non-territorial terminal phase males.

As in many other fishes, the number of ovulated eggs increases with female body size in bluehead wrasse (Shapiro et al., 1994). Furthermore, the number of eggs released

in a spawn is positively correlated with female body size, and the number of sperm a pair-spawning male releases is positively correlated with the size of his partner (Shapiro et al., 1994). Similarly, in bucktooth parrotfish, pair-spawning males released progressively more sperm with females in the medium, large and extra large size classes and increased the number of sperm released as a function of the number of eggs released (Marconato and Shapiro, 1996). Consequently, in a natural setting, it appears that pair-spawning male bluehead wrasse and bucktooth parrotfish use some cue associated with body size to allocate sperm during spawning.

In general, group-spawning initial phase male bluehead wrasse released six times more sperm per male than pair-spawning terminal phase males (Shapiro et al., 1994) suggesting that parasitic males do not modulate sperm release in relation to the number of competitors present. However, group-spawning males did increase the number of sperm released as a function of the number of eggs released (Shapiro et al., 1994) providing preliminary evidence that group-spawning males may differentially allocate sperm based on female size. In bucktooth parrotfish, streaking by parasitic males became more frequent as female size increased (Marconato and Shapiro, 1996).

The Japanese medaka (*Oryzias latipes*) has a mating system similar to the goldfish, insofar as males are promiscuous and do not provide parental care. Females can ovulate daily, but release all eggs in a single spawning act. Large *O. latipes* females spawn more eggs and males spend more time with larger females (Grant et al., 1995), suggesting that males can discriminate between females based on body size and use this information when courting. Because the goldfish mating system is promiscuous with an absence of parental care and the possibility for multiple spawning opportunities by both

sexes, I predict that males will actively compete for and allocate more sperm to spawning events with large females.

1.5. Mechanisms for Sperm Allocation in the Goldfish

Male goldfish may use visual or olfactory stimuli to assess the number of potential competitors or female fecundity. Immediately prior to and at the time of spawning, the reproductive behavior and physiology of male goldfish are affected by at least three types of chemical cues released by periovulatory females and mature males: 1) the preovulatory steroid pheromone, 2) the postovulatory prostaglandin pheromone and 3) unknown inhibitory and stimulatory cues from males (Stacey and Sorensen, 2002, 2006). The preovulatory steroid pheromone provides information about imminent ovulation whereas the postovulatory prostaglandin pheromone indicates presence of a receptive female; both pheromones induce both behavioral releaser and physiological primer effects in males (Stacey and Sorensen, 2002, 2006). The unknown cues from males have yet to be explored fully; however, males maintain low milt (sperm and seminal fluid) volume in the presence of males with basal levels of reproductive hormones, and increase milt volume in the presence of males with elevated hormone concentrations (Stacey et al., 2001; Fraser and Stacey, 2002). These cues from females and males appear to operate as a complex and dynamic information network (McGregor and Peake, 2000; Wisenden and Stacey, 2005) that strongly influences sperm allocation.

1.5.1. The Preovulatory Steroid Pheromone

The preovulatory pheromone appears to sexually prime male goldfish to prepare them for spawning. The major components of the preovulatory pheromone are the steroids 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), 17,20β-dihydroxy-4-pregnen-3-one-20-sulfate (17,20β-P-S) and androstenedione (AD). 17,20β-P, produced in large quantity by the follicle cells during the preovulatory surge of luteinizing hormone (LH), stimulates final oocyte maturation. Because nonovulatory females release minimal quantities of 17,20β-P and 17,20β-P-S, the dramatic increase in release of these steroids by periovulatory females enables them to serve as clear signals of imminent ovulation and spawning opportunities (Scott and Sorensen, 1994).

When exposed to even low (picomolar) concentrations of 17,20ß-P, male goldfish increase blood LH within minutes and milt volume in 4-6 h (Dulka et al., 1987; Zheng and Stacey, 1996). Following overnight 17,20ß-P exposure mimicking duration of exposure to an ovulatory female, males are more behaviorally competitive in spawning (DeFraipont and Sorensen, 1993), and achieve greater paternity during both competitive spawning and competitive *in vitro* fertilization, advantages that likely result from the combined effects of enhanced behavioral competitiveness, increased sperm release, and increased sperm motility (Zheng et al., 1997). Water-borne 17,20ß-P-S also increases male LH and milt, but differs from 17,20ß-P in the behavioral response it induces (Poling et al., 2001), in the olfactory receptors through which it acts, and in its release route (in urine rather than across the gill) (Sorensen et al., 1995a; Kobayashi et al., 2002). The function of AD, which can inhibit LH and milt in males (Stacey, 1991), is presently unclear (but see 1.5.3 below). However, it seems likely that AD (which is released with

17,20ß-P across the gill; Kobayashi et al., 2002) modulates male response to 17,20ß-P in the periovulatory period.

1.5.2. The Postovulatory Prostaglandin Pheromone

The postovulatory prostaglandin pheromone consists of prostaglandin $F_{2\alpha}$ (PGF) and its metabolite, 15-keto prostaglandin $F_{2\alpha}$ (15K-PGF) (Kobayashi et al., 2002). In female goldfish, the presence of eggs in the oviduct results in the production of PGF which acts in the brain to induce spawning behavior (Stacey, 1976; Kobayashi et al., 2002). Because PGF release increases dramatically at ovulation and rapidly decreases when all ovulated eggs are shed, it serves as a clear indicator of female sexual receptivity (Sorensen et al., 1988, 1995b; Kobayashi et al., 2002). Males respond to PGF and 15K-PGF by becoming immediately sexually active. Odors of PGF injected females and ovulated females produced an increase in male sex behaviors such as nudging and chasing (Sorensen et al., 1986). The resulting sexual interaction increases LH and milt (Kyle et al., 1985; Sorensen et al., 1989).

The preovulatory pheromone and the postovulatory prostaglandin pheromone increase milt volume by different mechanisms (Sorensen et al., 1989). 17,20β-P-induced milt increases are mediated by LH (Dulka et al., 1987). This mechanism requires at least 3 hours to increase milt volume at 20 °C and longer at lower temperatures (Dulka et al., 1987; Zheng and Stacey, 1996) whereas spawning stimuli increase milt volume in less than an hour (Kyle et al., 1985), a response which is relatively unaffected by temperature (Zheng and Stacey, 1996).

1.5.3. Male-Male Interactions in the Absence of Female Cues

Cues from males can both inhibit and stimulate milt production in other males, but these effects do not appear to be mediated by LH increase (Kobayashi et al., 2002). In the absence of stimulatory cues from males or ovulatory females, unstimulated males maintain low levels of milt in their sperm ducts and produce presumed chemical cues that suppress milt production in other males. For example, a male that is removed from a group of unstimulated males and isolated for 24 hours increases milt volume and serum testosterone levels but does not increase serum LH levels (Stacey et al., 2001). Once isolated males are returned to the unstimulated male group, milt volumes decline to the level of the grouped males after 24 hours (Fraser and Stacey, 2002). In contrast, if a male is either injected with human chorionic gonadotropin (hCG), or exposed to 17,20ß-P, and then placed in an unstimulated all-male group, the previously unstimulated males increase milt volume after 12 hours. Again, serum LH levels are unaffected by this response (Stacey et al., 2001). Thus, male related alterations in milt volume function by a different mechanism than 17,20β-P- or PGF- induced increases (Stacey, 2003).

The above results suggest that male goldfish might normally adjust their milt volume in response to the endocrine status of surrounding males. A surge of LH in a pre-ovulatory female will result in the release of 17,20β-P and exposed males will become stimulated. Unstimulated males that meet a stimulated male will respond by increasing milt volume and thus, respond indirectly to females.

1.5.4. Objectives of this Study

Currently, sperm allocation has not been studied in a non-guarding, externally fertilizing fish species. The goldfish system is a useful for practical reasons as they can be maintained in reproductive condition and spawn readily in the lab. Also, goldfish are involved in intense sperm competition, and the large body of information about the factors affecting milt production suggests that goldfish have evolved complex sperm allocation responses to social cues.

The objective of this study was to investigate sperm allocation in the male goldfish by determining 1) whether female size influences male mate choice behavior, 2) the effect of female size on milt and endocrine responses and 3) the effect of male competitors on milt and endocrine responses.

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2. EFFECTS OF FEMALE SIZE ON MALE MATE CHOICE IN GOLDFISH

2.1. Introduction

In externally fertilizing fishes, male mate choice has been investigated in a variety of higher taxa such as Atheriniformes (Pacific blue-eye, *Pseudomugil signifier*; Wong and Jennions, 2003; Wong et al., 2004), Beloniformes (Japanese rice fish, *Oryzias latipes*; Grant et al., 1995), Cypriniformes (zebrafish, *Danio rerio*; Pyron, 2003), Cyprinodontiformes (pupfish, *Cyprinodon macularius californiensis*; Loiselle, 1982), Gasterosteiformes (threespine stickleback, *Gasterosteus aculeatus*; Rowland, 1982; Sargent et al., 1986; Kraak and Bakker, 1998), Perciformes (redlip blenny, *Ophioblennius atlanticus*; Côte and Hunte, 1989: convict cichlid, *Cichlasoma nigrofasciatum*; Nuttall and Keenleyside, 1993: orangethroat darter, *Etheostoma spectabile*; Pyron, 1996: beaugregory damselfish, *Stegastes leucostictus*; Itzkowitz et al., 1998: sand goby, *Pomatoschistus minutus*; Kvarnemo and Forsgren, 2000: two-spotted goby, *Gobiusculus flavescens*; Amundsen and Forsgren, 2001; Pélabon et al., 2003: *Astatotilapia flaviijosephi*; Werner and Lotem, 2003) and Syngnathiformes (pipefish, *Nerophis ophidion*; Rosenqvist, 1990). The majority of these studies provide evidence that males have a preference for large females. In many fish species, including the common carp (*Cyprinus carpio;* Sivakumaran et al., 2003), a close relative of the goldfish (*Carassius auratus*), female body size is positively correlated with fecundity (Fuller, 1998; Michaletz, 1998; Jonsson and Jonsson, 1999; Loir et al., 2001; Wydoski, 2001; Pélabon et al., 2003). Because large females have greater fecundity, males should prefer to spawn with these females as it increases their reproductive fitness.

Behavioral laboratory studies indicate that males prefer larger females, which they might detect either by longer standard lengths or greater belly distention. In a number of visual choice experiments, for example, males were presented with two females of similar reproductive states but of differing lengths. Females were placed in different compartments where they could not see or interact with one another, and males indicated their choice of the larger female by 1) time in the response zone (Nuttall and Keenleyside, 1993; Grant et al., 1995; Werner and Lotem, 2003), 2) proximity to the compartment holding the female (Rosenqvist, 1990), or 3) courtship displays directed at each female (Sargent et al., 1986; Itzkowitz et al., 1998; Kraak and Bakker, 1998; Werner and Lotem, 2003; Wong and Jennions, 2003; Wong et al., 2004). Similarly, male threespine sticklebacks presented with female models that had either different belly distention or standard lengths performed more courtship displays towards the models with the greatest distention or largest standard length (Rowland, 1982, 1989). Further, male redlip blennies in a natural setting preferentially accepted larger females into their nests (Côte and Hunte, 1989).

Visual choice experiments, similar to those described above, also were conducted to evaluate the relative importance of female size and reproductive condition on male mate choice in the convict cichlid (*Cichlasoma nigrofasciatum*; Nuttall and Keenleyside, 1993). Female reproductive condition appears to be the primary determinant of male choice, because males chose large ovulated females over small ovulated females, and small ovulated females over large nonovulated females, but showed no preference when neither large nor small female were ovulated (Nuttal and Keenleyside, 1993).

In a few fish species, males showed either a weak preference or an absence of preference for the large female. Again, in a visual choice apparatus, male two-spotted gobies directed slightly more displays towards the large female (Pélabon et al., 2003), male sand gobies frequented the response zones of the females equally (Kvarnemo and Forsgren, 2000), and male zebrafish spent similar amounts of time with both females (Pyron, 2003). Additionally, in an experiment that permitted physical interaction between fish, male orangethroat darters mated with both the large and small female equally (Pyron, 1996). Possible explanations for the absence of choice include using fish from populations that appear to have a male biased sex ratio, such that males will court any female to which they are given access (Pyron, 1996; Kvarnemo and Forsgren, 2000), low variability in female fecundity (Pyron, 1996; Pélabon et al., 2003), and the importance of other visual cues in mate choice (Amundsen and Forsgren, 2001; Pélabon et al., 2003). Interestingly, male two-spotted gobies spent more time and directed more courtship displays towards colorful females (Amundsen and Forsgren, 2001) suggesting that they use reproductive coloration as their primary basis of mate choice.

Male mate choice has not been studied in a species like the goldfish where the mating system involves scramble competition and parental care is absent. Assuming that female goldfish exhibit a positive relationship between body size and fecundity, as has been shown in many other fishes, I predicted that males will prefer to mate with large females and therefore investigated whether males given a choice of two spawning partners spawn preferentially with the larger one, and whether this is influenced by the relative size of the male.

2.2. Methods

2.2.1 Experimental Animals

Goldfish of the comet variety were obtained annually from either Aquatic Imports (Calgary, Canada) or Hunting Creek Fisheries (Thurmont, Maryland, USA) between March and June, just prior to and during the spawning season. Fish were housed at the University of Alberta Aquatics Facility in 300 L holding tanks with a continuous inflow of dechlorinated water and fed flake food and koi pellets *ad libitum*. Goldfish were sexed based on the presence of tubercles on the opercula or pectoral fins of males and fish were kept in same sex groups of approximately 40. Holding tanks were maintained at a constant water temperature (17 °C) and photoperiod (16L:8D) and contained gravel and airstones, conditions intended to maintain males in a prespawning condition, with mature testes and capable of spawning and producing milt. Although it was intended that fish would be tested in the spring and summer they were purchased, disease in newly purchased fish, and the failure to obtain sufficient mature males in some years, resulted in fish often being tested a year or more after purchase. Experiments followed the guidelines set out by the Canadian Council on Animal Care.

2.2.2 Goldfish Reproductive Behavior

Normally, spawning occurs for about 1 hour after ovulation, and involves repeated entry into floating aquatic vegetation to deposit adhesive, unguarded eggs. In female goldfish, the presence of eggs in the oviduct results in the production of prostaglandin $F_{2\alpha}$ (PGF) which acts in the brain to induce spawning behavior (Stacey, 1976; Kobayashi et al., 2002). PGF is released with a metabolite, 15-keto-prostaglandin

$F_{2\alpha}$ (15K-PGF), which together act as a pheromone that induces male courtship behavior (Kobayashi et al., 2002). When all the eggs are shed, PGF synthesis drops dramatically and females become unreceptive and unattractive (Kobayashi et al., 2002). Nonovulated females injected with PGF perform normal spawning behavior (without oviposition) and are as attractive to males as are ovulated females (Stacey, 1981).

When a male goldfish encounters either an ovulated female or a nonovulated, PGF-injected female, he starts courting immediately. Courtship behavior consists of closely following or chasing the female and may result in physical contact with the female. When the male actively courts the ovulated or PGF-injected female and there is spawning substrate available, it usually results in sexual behavior (spawning). A *spawn* is a stereotyped set of behaviors in which the female and male ascend into the substrate, press together briefly, and thrash vigorously before leaving the substrate. If the female is ovulated, then she deposits her eggs onto the substrate and the male releases sperm over the eggs. Because it is the female who initiates the ascent into the substrate and the male who follows her, spawns are determined by the female. Interestingly, the spawning rate is highly variable among females but quite consistent within females (Kobayashi and Stacey, 1993).

Sometimes the female and male ascend into the substrate and leave without spawning. This behavior, termed a *rise together*, might occur due to inappropriate substrate microenvironment, misalignment of the spawning pair, or partner rejection by one or both fish.

Additionally, an uncourted female may enter the substrate without the male and this behavior is termed a *rise alone*. This behavior may function to advertise female

receptivity and to incite male responsiveness. Also, rise alone behavior is useful in assessing female receptivity (the likelihood that she would spawn) in cases where the male is not courting.

2.2.3. Experimental Protocol

Fish from holding tanks were anesthetized in 0.05% 2-phenoxyethanol (Syndel, Vancouver, B.C.) and weighed to obtain experimental spawning trios (two females and one male) for three treatment groups: *smaller* (male smaller than both females), *intermediate* (male between the female weights) and *larger* (male larger than both females) (Table 2.1). Males in these three treatment groups are referred to as smaller, intermediate, or larger and these terms refer not to the males' absolute size, but rather to their size relative to the females with which they were tested. Because the limited number of suitable fish did not allow different females to be used for each behavioral test, a set of experimental females was selected and held as pairs (one large and one small) that were tested repeatedly, each time with a different male. Experimental males, which were tested only once, were selected on the basis of breeding tubercles on the opercula and pectoral fins.

For each behavioral test, one male and two females were placed for at least 4 days in an aerated, flow-through 300 L experimental tank containing gravel and spawning substrate (two artificial plants constructed of green acrylic yarn and suspended on the water's surface). In the morning, immediately prior to each test, females were netted, given an intra-muscular injection of PGF (Lutalyse, Upjohn; 200 ng per g body weight) to induce spawning behavior (Kobayashi et al., 2002), and immediately returned to the tank. The onset of chasing behavior by the male (typically within 15 minutes of PGF

injection) marked the beginning of a 60 minute recording session, during which behaviors were recorded continuously for the first nine minutes in each of six, 10 minute periods. Recorded behaviors included the incidence of spawn, rise together and rise alone, and the duration of courtship (time spent chasing, performing spawns, or performing rise togethers) that the male directed towards each female.

Behavioral tests were included in the data set if there was a minimum of 10 spawns and 10 rises alone (combined totals of both females) and if both females performed at least one of these behaviors. Rising alone was included in the criterion in an effort to include only tests where both females showed receptivity. Of 80 tests conducted, 64 met the minimum criterion (smaller males, $N = 23$; intermediate males, $N = 23$; larger males, $N = 18$). Twelve of the sixteen excluded trials involved the same four pairs of unresponsive females.

2.2.3 Statistics

Body weight data (log transformed) and courtship duration (arcsine transformed) were analyzed using standard parametric statistics (ANOVA, Tukey-Kramer Multiple Comparisons tests, one sample *t* tests; Instat V. 3, Graphpad Software, Inc., San Diego, CA, USA). Behavioral frequency data (spawns, rise together, and rise alone) were analyzed using non-parametric statistics (Wilcoxon matched-pairs signed-ranks test, Kruskal Wallis test, Friedman test; Instat V. 3, Graphpad Software, Inc., San Diego, CA, USA). All statistical tests were two-tailed and evaluated at the $\alpha = 0.05$ significance level.

2.3. Results

2.3.1. Body Sizes and Spawning Activities of the Three Treatment Groups

Body weight of smaller males was significantly less than that of intermediate and larger males (ANOVA, $F_{2,61} = 8.070$, $P = 0.0008$; Tukey-Kramer test, smaller males vs. intermediate males, $P < 0.05$, smaller males vs. larger males, $P < 0.01$), which did not differ significantly (Tukey-Kramer test, intermediate males vs. larger males, $P > 0.05$; Table 2.1). Although the same set of females was used in behavioral tests with smaller, intermediate and larger males, the mean weights of females were not equal among the three treatments because some tests that did not meet the minimum spawning criterion were omitted.

To determine if any differential responses of smaller, intermediate and larger males towards large and small females are indicative of male preference, it first is important to determine whether the results support two assumptions implicit in the experimental design: 1) that smaller, intermediate and larger males engage in equivalent levels of reproductive activity; 2) that reproductive activity of females is not influenced by male size. The first assumption is supported because, as in previous studies (Stacey and Kyle, 1983), all males began to court within 15 minutes of being exposed to PGF-injected females, courted almost continuously throughout the observation period, and engaged in comparable amounts of total spawning (Kruskal Wallis test, $H = 4.942$, $P = 0.085$, smaller and intermediate male groups, $N = 23$, larger males, $N = 18$) and total rise together activity (Kruskall Wallis test, $H = 4.797$, $P = 0.091$, smaller and intermediate male groups, $N = 23$, larger males, $N = 18$; Figure 2.1). The second assumption also appears to be met because, at least for the twelve female pairs that were tested with all

three male sizes, spawning and rising behaviors of both small and large females were unaffected by male size (Friedman test, small female spawning behavior, $\chi^2_r = 2.681$, $P = 0.262$, large female spawning behavior, $\chi^2_r = 0.043$, $P = 0.979$, small female rise together behavior, $\chi^2_r = 2.085$, $P = 0.353$, large female rise together behavior, $\chi^2_r = 3.167$, $P = 0.205$, $N = 12$ in each test; Figure 2.2). Although large and small females differed in terms of both spawning (see 2.3.3 below) and rise alone behaviors (see 2.3.2 below), they did not differ in their total number of entries (spawn + rise together + rise alone) into the spawning substrate (Wilcoxon test, females engaging in activity with smaller males, $T_{12} = 14.000$, $P = 0.622$, intermediate males, $T_{12} = 26.000$, $P = 0.339$, larger males, $T_{12} = 20.000$, $P = 0.470$; Figure 2.2D). Based on these findings, I assume that the following measures of male activity represent choice by males rather than by females.

2.3.2. Courtship Behavior

Although smaller, intermediate and larger males courted the large female more than the small female, the difference was significant only for the intermediate and larger males (one sample t test, smaller males, $t = 1.190$, df = 22, $P = 0.247$, intermediate males, $t = 3.876$, df = 22, $P = 0.0008$, larger males, $t = 3.243$, df = 17, $P = 0.005$; Figure 2.3A). Also, there was no clear trend for males to increase or decrease their courtship of large females throughout the test (repeated measures ANOVA, smaller males, $F_{5,110} = 0.949$, $P = 0.452$, intermediate males, $F_{5,110} = 1.100$, $P = 0.365$, larger males, $F_{5,85} = 1.266$, $P = 0.286$; Figure 2.3B).

It appears that the males' preferential courtship of large females (Figure 2.3A) increased rise alone behaviors by small females (Figure 2.2C). Small females performed

more rise alone behavior than did large females, whether in the presence of smaller, intermediate or larger males (Figure 2.2C); however, the difference was significant only for females interacting with intermediate males (Wilcoxon test, females interacting with smaller males, $T_{12} = -45.000$, $P = 0.077$, intermediate males, $T_{12} = -64.000$, $P = 0.009$, larger males, $T_{12} = -39.000$, $P = 0.083$). These findings support the assumption that rise alone behavior is indicative of female intention to spawn.

2.3.3. Spawning Behavior

The total activity (spawn or rise together) did not differ among the three male groups (Figure 2.1). However, whereas smaller males performed equivalent numbers of spawning acts with small and large females (Wilcoxon test, $T_{23} = 15.000$, $P = 0.006$; Figure 2.4A), both intermediate and larger males performed more spawning behavior with the large female than with the small female, although only for intermediate males was the difference significant (Wilcoxon test, intermediate males, $T_{23} = 157.000$, $P = 0.009$, larger males, $T_{18} = 78.000$, $P = 0.064$; Figure 2.4A). The same trends were observed in the rise together behaviors of the three male groups (Wilcoxon test, smaller males, $T_{23} = -5.000$, $P = 0.949$, intermediate males, $T_{23} = 160.000$, $P = 0.007$, larger males, $T_{18} = 87.000$, $P = 0.021$; Figure 2.4B).

The proportion of spawns with the large female are positively correlated with the proportion of courtship directed towards the large female for smaller, intermediate and larger males (Spearman rank correlation, smaller males, $r = 0.824$, $P < 0.0001$, $N = 23$, intermediate males, $r = 0.603$, $P = 0.002$, $N = 23$, larger males, $r = 0.668$, $P = 0.003$, $N = 18$; Figure 2.5).

Male preference for spawning with large females was not consistent throughout the 60 minute test. Smaller males, which showed no spawning preference for large females, also exhibited no preference throughout the test (Wilcoxon test, 0-30 minutes, T_{23} = -13.000, P = 0.849, 30-60 minutes, T_{23} = 22.000, P = 0.754; Figure 2.6A). For intermediate and larger males, however, preference was generally more pronounced in the latter 30 minutes (Wilcoxon test, intermediate males, T_{23} = 169.000, P = 0.005; larger males, T_{18} = 97.000, P = 0.020; Figure 2.6B, C). Only for intermediate males did preference for the large female approach significance in the first half of the test (Wilcoxon test, intermediate males, T_{23} = 119.000, P = 0.054, larger males, T_{18} = 57.000, P = 0.144; Figure 2.6B, C).

The results for the rise together behavioral data are quite similar to the spawn data (Figure 2.7). In the first half of the test, smaller, intermediate and larger males performed similar amounts of rising behavior with large and small females (Wilcoxon test, smaller males, T_{23} = -26.000, P = 0.709, intermediate males, T_{23} = 78.000, P = 0.123, larger males, T_{18} = 53.000, P = 0.225; Figure 2.7). In the second half, smaller males continued this trend (Wilcoxon test, T_{23} = 2.000, P = 0.973; Figure 2.7A), whereas intermediate and larger males rose more with the large female than with the small female (Wilcoxon tests, intermediate males, T_{23} = 142.000, P = 0.030, larger males, T_{18} = 91.000, P = 0.048; Figure 2.7B, C).

In terms of their courtship (Figure 2.3), spawning and rising behaviors (Figure 2.4), intermediate and larger males chose large females over small females. Therefore, it might be expected that the strength of this choice would increase as the difference between female size increases. However, only courtship by larger males was

significantly correlated with female size differential, and this was dependent on one outlying data point (Spearman rank correlation, $r = 0.548$, $P = 0.019$, $N = 18$; Figure 2.8).

2.4. Discussion

This study appears to be the first to examine male mate choice not only in a non-guarding species that employs scramble competition, but also in situations where the male is either larger or smaller than both females. The results demonstrate that male goldfish prefer to court and spawn with larger females, and also show that this preference is influenced by relative body size. Thus, when allowed to interact simultaneously with two female spawning partners, males exhibited no preference if they were smaller than both females, but preferred the larger of two females if they were larger than at least one of the females.

For two reasons, it seems likely that this experiment reveals a mate choice that male goldfish exhibit in natural conditions. First, goldfish live in unstructured groups (Magurran, 1984) in which males would be expected to have the opportunity to spawn with females of various sizes. Second, ovulation is synchronous (Kobayashi et al., 1988, 2002), so males have a simultaneous choice among females.

In this experiment, male goldfish in the intermediate and larger treatment groups spent more courtship time (Figure 2.3A) and performed more spawns with the larger female (Figure 2.4A). Assuming that female size is positively correlated with fecundity in the goldfish, as occurs in many other fish species (Fuller, 1998; Michaletz, 1998; Jonsson and Jonsson, 1999; Loir et al., 2001; Wydoski, 2001; Pélabon et al., 2003; Sivakumaran et al., 2003) intermediate and larger males should preferentially court the large female because she represents the potential to fertilize a greater number of eggs,

thereby directly increasing the male's reproductive success. This result is in agreement with many other studies investigating male mate choice in externally fertilizing fishes (Sargent et al., 1986; Rosenqvist, 1990; Nuttall and Keenleyside, 1993; Grant et al., 1995; Werner and Lotem, 2003; Wong and Jennions, 2003).

Also, in some species, larger females produce larger eggs (Kraak and Bakker, 1998; Dickerson, et al., 2002; Sivakumaran et al., 2003). If large female goldfish produce bigger eggs, then males may increase reproductive success by producing better quality offspring. The effect of maternal investment into the size at emergence and survivability of the offspring has been investigated in a variety of salmonid fishes (Beacham and Murray, 1990; Kristjánsson and Vøllestad, 1996; Einum and Fleming, 1999; Heath et al., 1999; Vøllestad and Lillehammer, 2000). In the brown trout (*Salmo trutta*), egg size was positively correlated with the length at hatching and life span (Vøllestad and Lillehammer, 2000) and research conducted under semi-natural conditions suggests that juveniles hatched from large eggs experience greater growth and lower mortality than their small-egg counterparts (Einum and Fleming, 1999).

Interestingly, the three male treatment groups performed equivalent spawns with large females (Figure 2.4A). If females have an upper limit of spawning frequency, then all male treatment groups appeared to have engaged in sufficient spawning activity to reach this limit with large females. The difference among the groups is in the spawning behavior with the small females. It appears that smaller males spawn with both small and large females at the upper limit of frequency, whereas intermediate and larger males fail to spawn with small females at this proposed upper limit, likely because they spend

significantly more time courting large females (Figure 2.3A), and thus forego some spawning opportunities with the small females.

The results from the smaller, intermediate and larger treatments clearly indicate that male preference depends on relative body size, although the explanation for this finding is unclear. These experiments were done in the absence of competition, allowing males to express their mate choice preferences without restriction. Smaller males did not show a preference, whereas intermediate and larger males appear to discriminate against the smallest female. In nature, where goldfish live in unstructured groups (Magurran, 1984) with individuals of varying size, male mate choice is probably influenced by competition from other males, with the result that small males may not get many opportunities to spawn with bigger females. Larger males are able to position themselves closer to the female because they can swim at faster speeds (Bainbridge, 1958) and because they can displace smaller males by their physical presence (personal observation). Thus, males likely spawn with individuals of similar size, although they maintain a preference for larger females in the absence of competition.

A possible mechanism for discriminating female size may be to use tactile stimuli, which may be more reliable than visual stimuli, given that goldfish and related carps typically inhabit turbid waters and that ovulation occurs at night (Kobayashi et al., 2002). Early in the testing period, intermediate and larger males performed equivalent spawning and rising behaviors with the two females but performed increasingly more spawning and rising behavior with large females as the testing period progressed (Figure 2.6C, 2.7C). Therefore, males may need to engage in sexual behavior with both females in order to gain more information through tactile stimuli. However, it is unclear how this

would apply to natural situations where males are unlikely to have an extended opportunity to "compare" females.

Similar to male mate choice experiments conducted in the field (Côte and Hunte, 1989; Itzkowitz et al., 1998), this study allowed males and females to interact physically during the observation period. I believe that this gave a better indication of mate choice because the frequency of sexual behavior was measured directly. In deference to the multitude of studies using partitioned, three chambered tanks (Sargent et al., 1986; Itzkowitz et al., 1998; Kraak and Bakker, 1998; Werner and Lotem, 2003; Wong and Jennions, 2003; Wong et al., 2004), this alternative design was considered. However, the partitioned, three chambered tank design was not ideal because male goldfish do not produce distinct courtship displays when separated from a female by a barrier, and male choice would have been measured indirectly as the time spent in the response zone.

Despite the clear results, without considering competition, the spawning situation in this experiment is unnatural. Further investigations should focus on determining how the pattern of mate choice observed in the absence competition is affected by the number and size of competitors.

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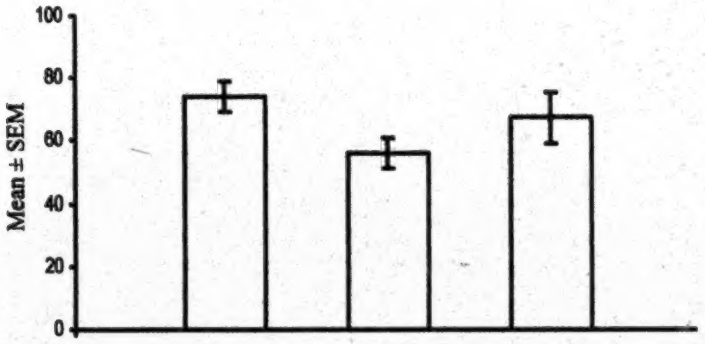
Table 2.1. Body weight (mean ± SEM; range) of goldfish used in male smaller, intermediate and larger treatment groups.

Male Treatment	Male Weight (g)	Female Weight (g)		Mean Difference Between Male	
		Small	Large	& Small Female	& Large Female
Smaller	57.8 ± 4.5* (31.4 – 103.5)	65.1 ± 4.8 (35.4 – 109.9)	76.5 ± 5.0 (40.9 – 120.5)	18.7 ± 1.7 (6.2 – 39.5)	7.3 ± 1.0 (0.2 – 17.8)
Intermediate	75.1 ± 4.5 (42.8 – 117.3)	66.3 ± 4.8 (33.2 – 113.7)	81.1 ± 4.6 (44.66 – 120.6)	6.0 ± 0.8 (1.2 – 13.3)	- 8.9 ± 1.6 - (31.1 – 1.5)
Larger	83.9 ± 6.3 (46.5 – 138.8)	65.4 ± 5.7 (37.6 – 109.9)	76.6 ± 6.1 (40.9 – 120.5)	- 7.3 ± 1.2 - (21.4 – 0.5)	- 18.5 ± 1.7 - (30.9 – 8.9)

* significantly smaller than medium and large males ($P < 0.05$)

A. Spawn



B. Rise Together

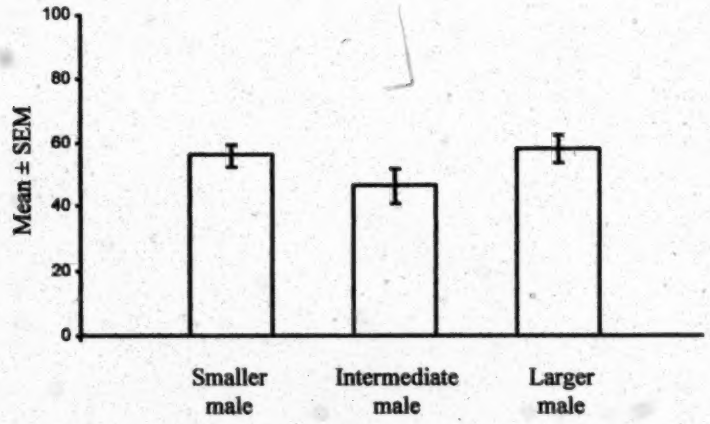


Figure 2.1. Total spawn and rise together behaviors (mean ± SEM) with both large and small females by males in the smaller ($N = 23$), intermediate ($N = 23$) and larger male groups ($N = 18$): (A) spawns and (B) rise together.

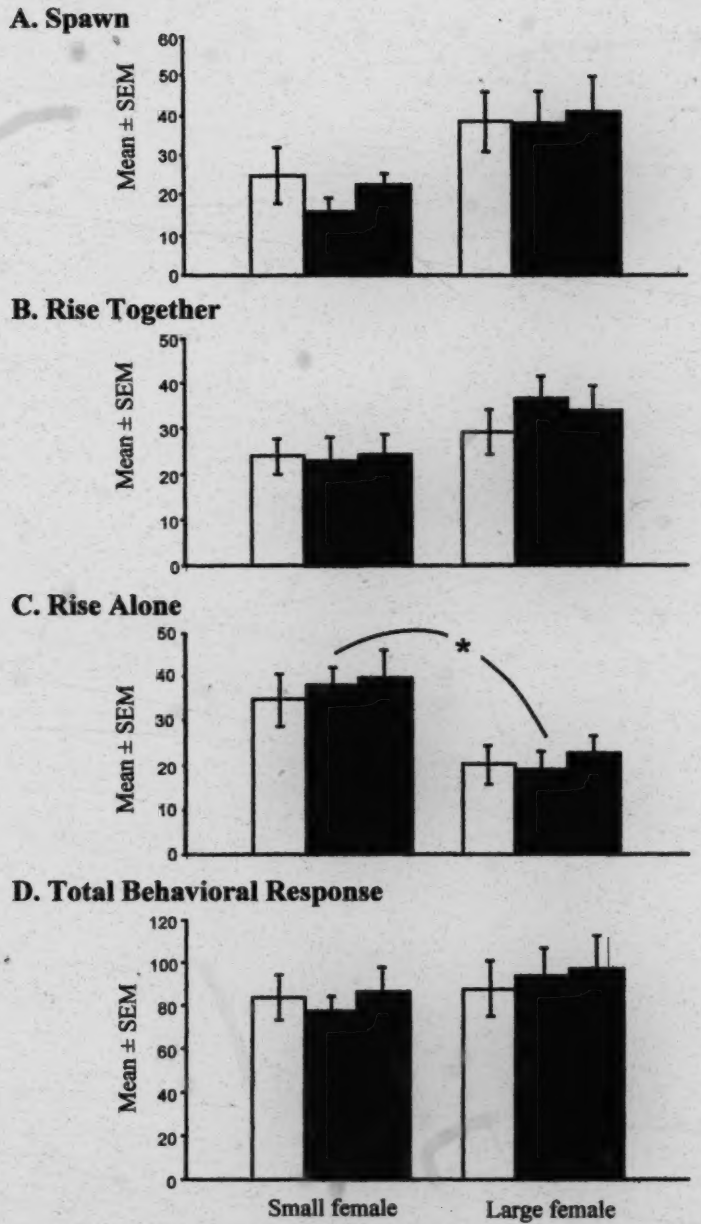
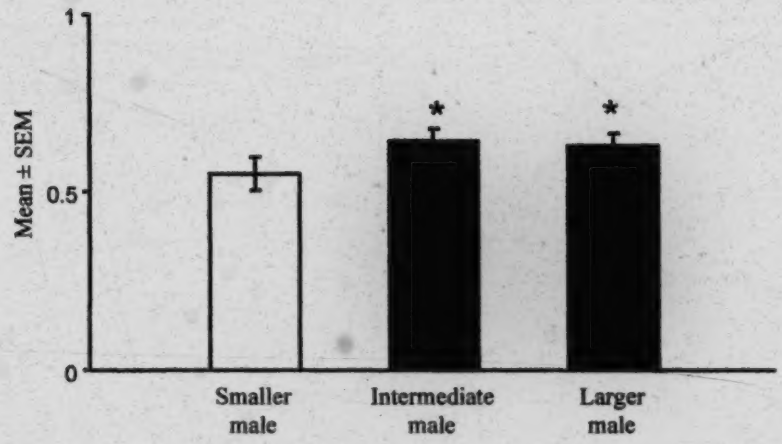


Figure 2.2. Large and small female behaviors (mean ± SEM; $N = 12$ per group) with smaller (□), intermediate (▒) and larger (■) males for (A) spawns, (B) rise together, (C) rise alone and (D) total behavioral response. * = $P < 0.05$.

A.



B.

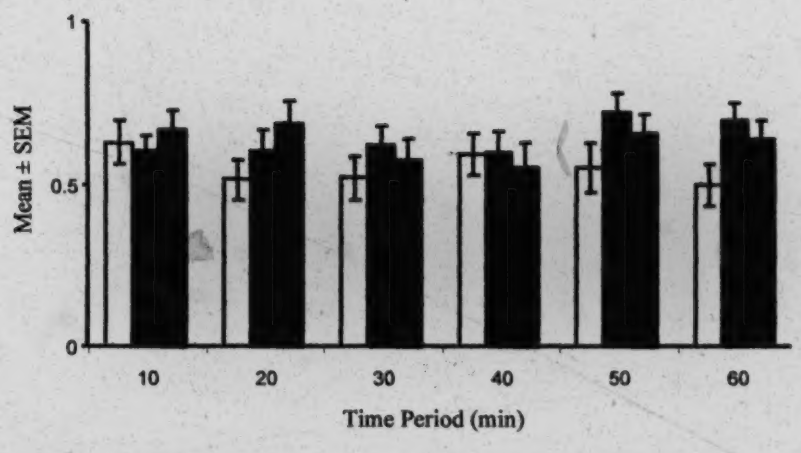
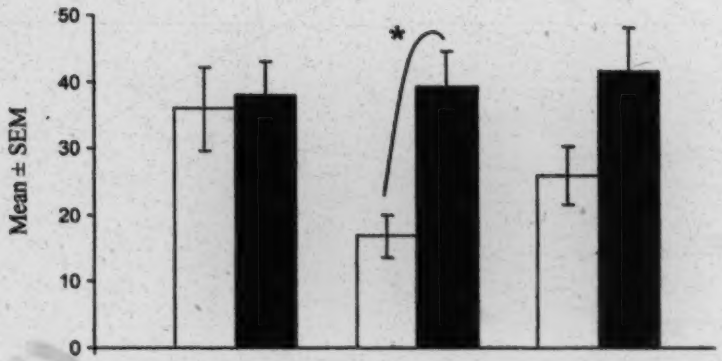


Figure 2.3. Proportion of male courtship time directed towards the large female (mean ± SEM) by the smaller (□; $N = 23$), intermediate (■; $N = 23$) and larger (■; $N = 18$) male groups in (A) 60 minutes and (B) each 10 minute observation period. * = $P < 0.05$.

A. Spawn



B. Rise Together

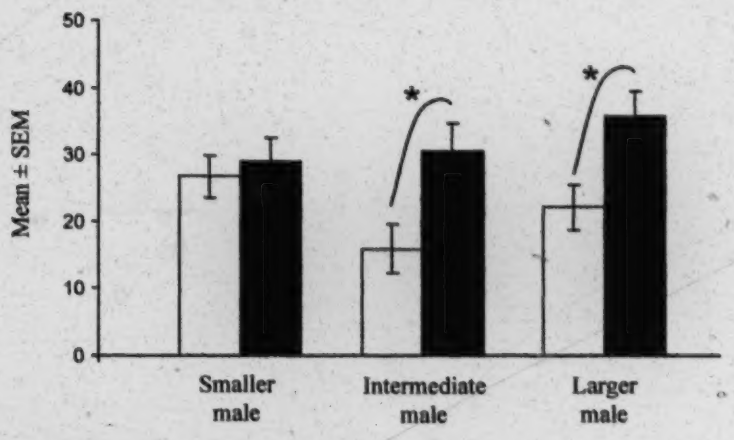
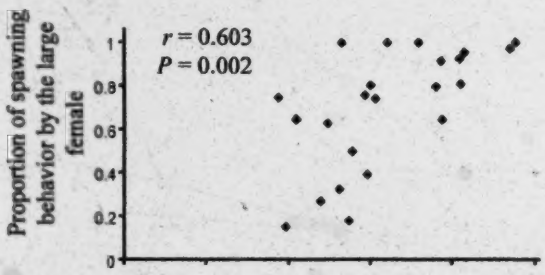


Figure 2.4. (A) spawns and (B) rises together (mean ± SEM) with small (□) and large females (■) by males in the smaller ($N = 23$), intermediate ($N = 23$) and larger ($N = 18$) male groups. * = $P < 0.05$.

A. Smaller males

B. Intermediate males



C. Larger males

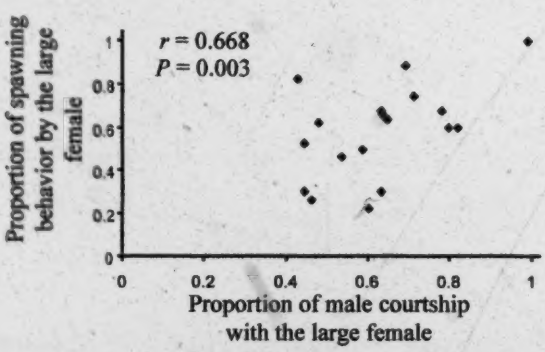


Figure 2.5. Relationship between the proportion of spawning by the large females and the proportion of courtship males directed towards large females for (A) smaller ($N = 23$), (B) intermediate ($N = 23$) and (C) larger ($N = 18$) males. r and P values from Spearman rank correlation.

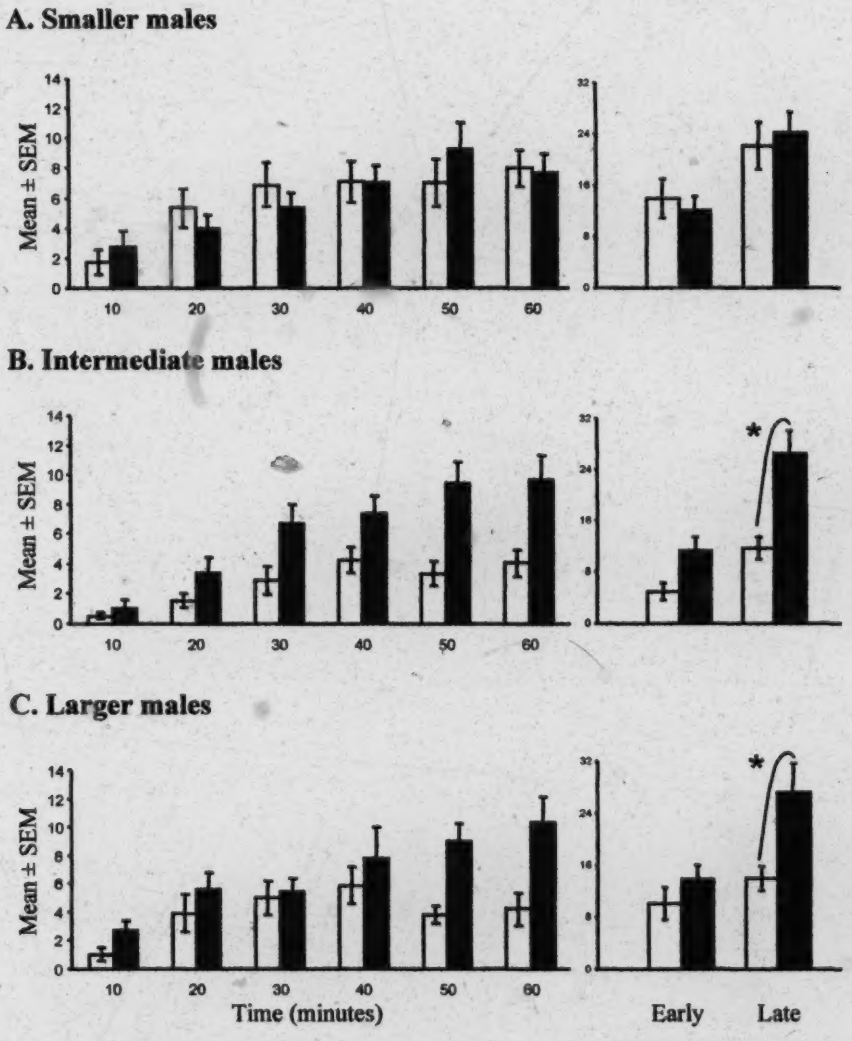


Figure 2.6. Spawning frequency (mean ± SEM) of the small (□) and large female (■) with (A) smaller ($N = 23$), (B) intermediate ($N = 23$) and (C) larger ($N = 18$) males during the 60 minute observation period and during the pooled early (0-30 minutes) and late (30-60 minute) periods. * = $P < 0.05$; Wilcoxon test.

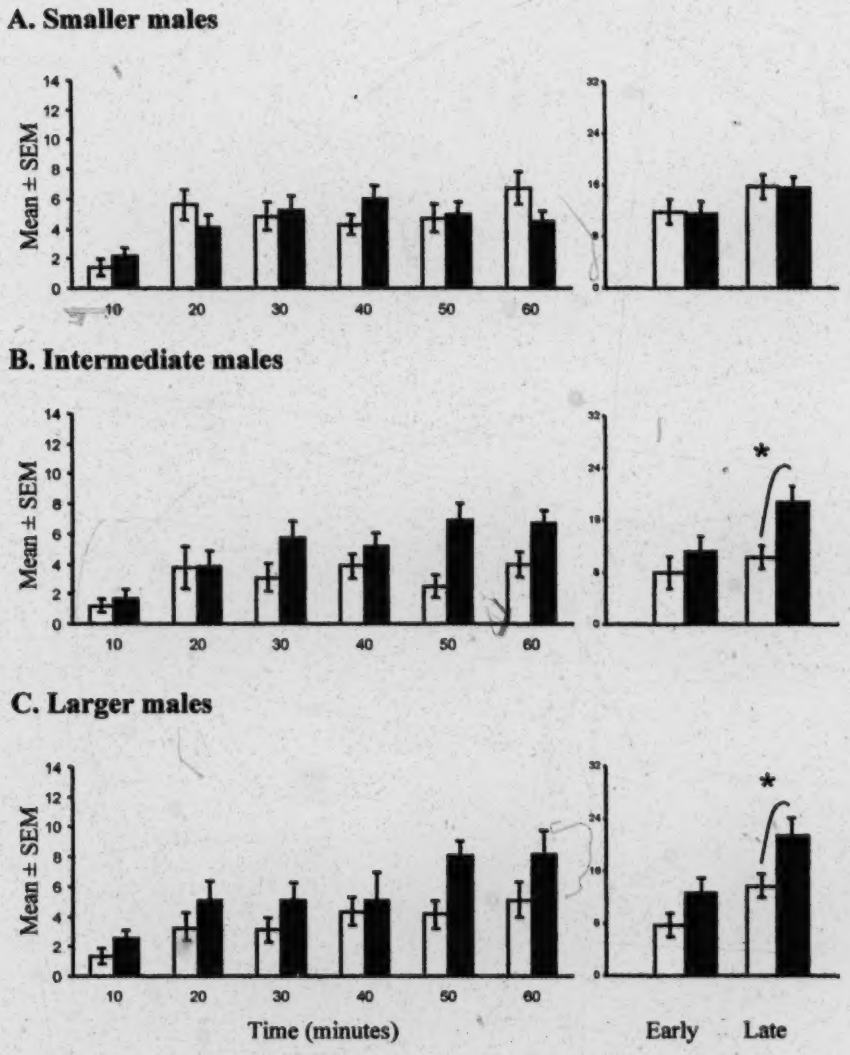


Figure 2.7. Rise together frequency (mean ± SEM) of the small (□) and large female (■) with (A) smaller ($N = 23$), (B) intermediate ($N = 23$) and (C) larger ($N = 18$) males during the 60 minute observation period and during the pooled early (0-30 minutes) and late (30-60 minute) periods. * = $P < 0.05$; Wilcoxon test.

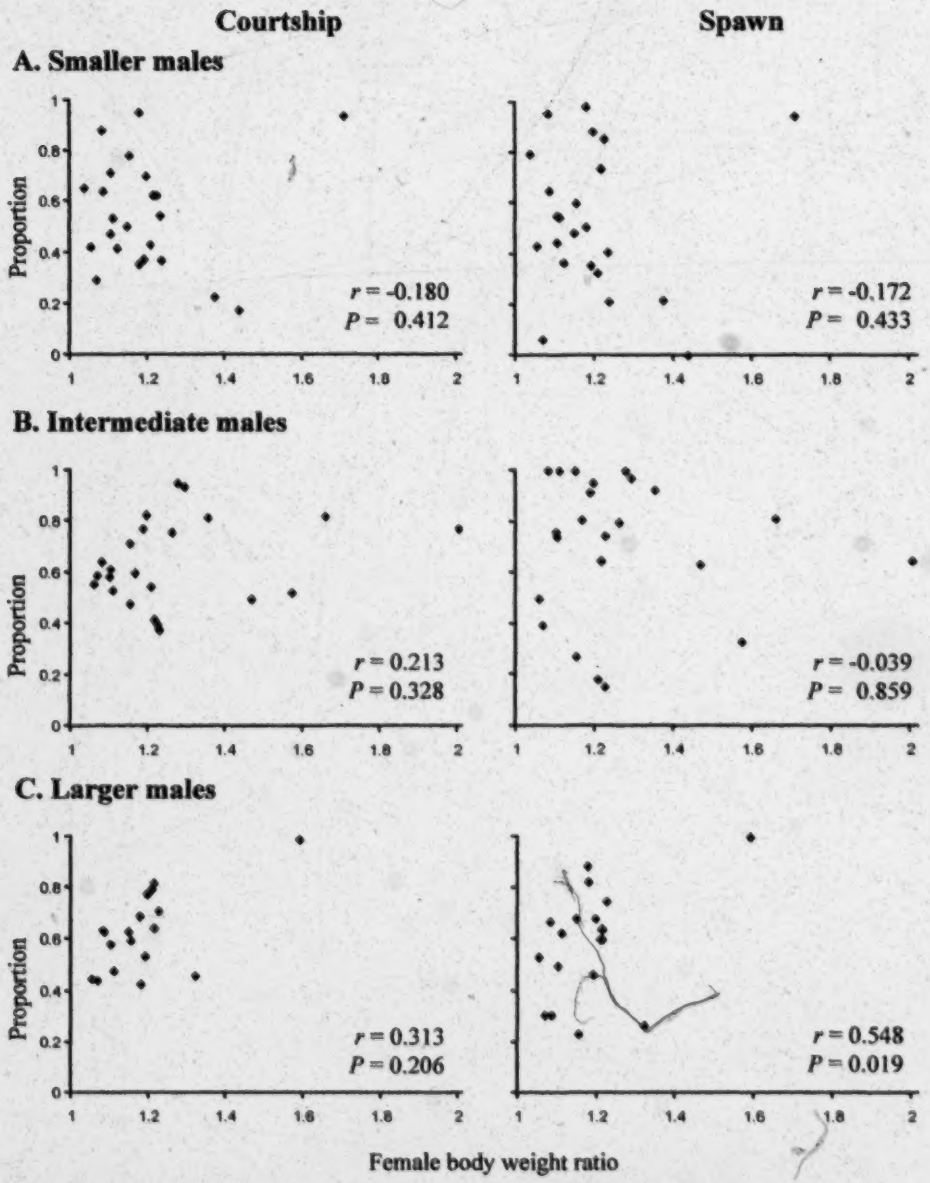


Figure 2.8. Relationship between the relative size of the large and small females and the proportion of courtship or spawning with the large female for (A) smaller ($N = 23$), (B) intermediate ($N = 23$) and (C) larger males ($N = 18$). r and P values from Spearman rank correlation.

3. EFFECT OF FEMALE SIZE ON MILT VOLUME, LUTEINIZING HORMONE AND TESTOSTERONE

3.1 Introduction

Sperm allocation is a male reproductive strategy that attempts to maximize reproductive success over the entire mating period. It is expected that males will allocate sperm differentially to reproductive events because the cost of producing sperm is non-trivial (Dewsbury, 1982) and because, in many species of fish, female body size is positively correlated with fecundity (Shapiro et al., 1994; Fuller, 1998; Michaletz, 1998; Jonsson and Jonsson, 1999; Loir et al., 2001; Wydoski, 2001; Pélabon et al., 2003; Sivakumaran et al., 2003). The effect of female body size on sperm allocation in fishes is a relatively new area of research. Currently, the few studies available are focused on a limited number of fish species such as the bluehead wrasse (*Thalassoma bifasciatum*; Shapiro et al., 1994), the bucktooth parrotfish (*Sparisoma radians*; Marconato and Shapiro, 1996), the three-spined stickleback (*Gasterosteus aculeatus*; Zbinden et al., 2001) and the sailfin molly (*Poecilia latipinna*; Aspbury and Gabor, 2004).

The most comprehensive research on female size and sperm allocation involves wild bluehead wrasse (Shapiro et al., 1994) and bucktooth parrotfish (Marconato and Shapiro, 1996). Both species are protogynous hermaphrodites with external fertilization. In bluehead wrasse, where the number of eggs stripped from females was positively correlated with total length, number of sperm release was positively correlated with female size (Shapiro et al., 1994). Similarly, in bucktooth parrotfish, sperm release in pair spawning males was positively correlated both with female size and with the

numbers of eggs released (Marconato and Shapiro, 1996). In the internally fertilizing sailfin molly, a seven day interaction with large females induced more sperm production (priming effect) than did interaction with small females (Aspbury and Gabor, 2004), indicating that female body size influences sperm allocation.

In contrast, ejaculate size in three-spined sticklebacks does not correlate with female weight, standard length or estimated egg mass (Zbinden et al., 2001). Because spermatogenesis is inhibited during the breeding season, it is expected that males would carefully partition sperm investment. However, males release sperm into an enclosed nest that, in comparison to open water spawners, limits dispersion. Additionally, sperm competition may be low because other males are restricted from entering the nest.

Chapter 2 provided behavioral evidence that male goldfish (*Carassius auratus*) spawn preferentially with larger females. Therefore, as in *T. bifasciatum* and *S. radians*, male goldfish may have a physiological mechanism to allocate more sperm to spawning encounters with large females. In addition, males should carefully allocate sperm to reproductive events because the goldfish mating system is promiscuous and females ovulate in synchrony (Kobayashi et al., 2002). Thus, it is realistic to expect that males will have the opportunity to mate with multiple females on the same day, as well as over the course of the breeding season. Although the effect of female body size on sperm allocation has not been studied in the goldfish, many studies have shown that spawning interactions affect both milt (sperm and seminal fluid) volume and luteinizing hormone (LH), a major sex hormone (Kyle et al., 1985; Sorensen et al., 1989). Therefore, it seemed reasonable to investigate whether the magnitude of these responses might be affected by female body size.

3.2 Methods

3.2.1 Experimental Animals

Goldfish of the comet variety were obtained annually from either Aquatic Imports (Calgary, Canada) or Huntington Creek (Maryland, USA). Shipments were made in the springtime between March and June, just prior to the reproductive season. Fish were housed at the University of Alberta Aquatics Facility in 300 L holding tanks with a continuous inflow of dechlorinated water. Holding tanks were maintained at a constant water temperature (17 °C) and photoperiod (16L:8D) and contained gravel and airstones. Fish were kept in same sex groups of approximately 40. Male goldfish were sexed based on the presence of tubercles on the operculum or on the pectoral fins. Fish were fed flake food and koi pellets *ad libitum*. Experiments followed the guidelines set out by the Canadian Council on Animal Care.

3.2.2 Experimental Protocol

The two experiments described below were conducted to determine whether the magnitudes of milt and endocrine responses to courtship were influenced by relative female size. Male goldfish courted prostaglandin $F_{2\alpha}$ (PGF)-injected females for 30 minutes in experiment 1 and 90 minutes in experiment 2. It was important to investigate short and long time periods because males may vary in their response to small and large females by altering the rate of filling or the duration of filling of the sperm ducts. Differences in the rate of filling should be apparent after 30 minutes of courtship because treatment males will not have time to fill sperm ducts to capacity and, thus, rate-of-filling effects should be evident. Alternatively, it is possible that males courting large females

may fill the sperm ducts at the same rate but for a longer duration than males courting small females. Difference in the duration of filling should be apparent after 90 minutes.

Experiment 1: 30 minute courtship

The experiment involved 3 male treatment groups, which were stripped of milt immediately prior to and following either a 30 minute period of courtship or a 30 minute period of isolation. Four to five days prior to experimental testing, 36 males of similar weight were placed in 60 L holding tanks in groups of three and 24 females were placed in 60 L holding tanks in groups containing three small females (33.0 ± 1.9 g) and three large females (109.2 ± 3.8 g). To ensure that males held similar milt volumes in the sperm ducts prior to treatment, males were stripped of milt immediately prior to testing as described by Stacey et al. (2001). Briefly, males were anaesthetized (2-phenoxyethanol; 0.05%, Syndel, Vancouver, Canada) and placed upside down in a moist, slotted foam pad. Gentle pressure was applied to the abdominal area, just anterior to the gonopore and milt was drawn into pre-weighed hematocrit tubes until milt could no longer be expressed. Males were revived and returned to their holding aquaria. To induce receptive behavior, females received an intra-muscular injection of PGF at an approximate dose of 200 ng per g body weight and were placed individually in 60 L testing aquaria which contained gravel and an air stone. Experiments were conducted in the absence of spawning substrate to minimize the chance that the male would release milt during the test period. From a single holding tank containing three males, one was transferred to a test tank containing a female larger than the male, the second was transferred to a test tank containing a female smaller than the male, and the third (control) male remained in

the holding tank. The 30 minute test period for each male began with his first display of chasing (courtship) behavior. After 30 minutes of courting, the two courting males and the control male were anaesthetized, stripped of milt a second time, bled from the caudal vasculature by syringe, and returned to their holding tanks. The hematocrit tubes were weighed and the calculated milt sample weights were expressed as volumes, assuming a milt density of 1.0 g/ml.

Blood samples were allowed to clot on ice for 3-4 hours before being centrifuged. Serum was transferred to a new centrifuge tube with a preservative (1 µl thimerosol; 1%), frozen on dry ice and stored at –20 °C. Serum LH was determined by a carp radioimmunoassay (RIA) as described by Peter et al. (1984). Serum testosterone concentration (T) was not assessed because blood serum volume was limited.

Experiment 2: 90 minute courtship

This experiment was conducted in the same way as experiment 1, except that fish were allowed to court for 90 minutes before milt and blood samples were taken. Additionally, serum testosterone was determined by an enzyme linked immunosorbent assay (ELISA) kit (MP Biomedicals, Orangeburg, NY, USA) according to the manufacturer's instructions.

3.2.3 Statistics

Body weight data and serum LH concentrations were analyzed by ANOVA and Tukey-Kramer Multiple Comparisons tests (Instat V. 3, Graphpad Software, Inc., San Diego, CA, USA). Milt volumes and serum T concentrations could not be normalized by

transformation, and therefore were analyzed by Kruskal Wallis test. Milt volume pre-test and post-test differences within groups were analyzed by Wilcoxon-matched pairs signed-ranks test (Instat V. 3, Graphpad Software, Inc., San Diego, CA, USA). All statistical tests were two-tailed and evaluated at the $\alpha = 0.05$ significance level.

3.3 Results

3.3.1 Experiment 1: 30 Minute Courtship

Male body weights did not differ among the three treatment groups (ANOVA, $F_{2,32} = 0.177$, $P = 0.839$; Table 3.1), which also had equivalent milt volumes prior to the test (Kruskal-Wallis test, $H = 1.491$, $P = 0.475$, small and large male groups, $N = 12$, controls, $N = 11$; Figure 3.1A). Post-test milt volumes were smaller than pre-test volumes in control males (Wilcoxon test, $T_{11} = 66.000$, $P = 0.001$) but not in the small or large male groups (Wilcoxon test, small male, $T_{12} = 48.000$, $P = 0.640$, large male, $T_{12} = 20.000$, $P = 0.470$; Figure 3.1A). Post-test milt volumes differed among groups (Kruskal-Wallis test, $H = 10.296$, $P = 0.006$, small and large male groups, $N = 12$, controls, $N = 11$) and were larger in the courting groups than in the control group (Dunn's test, $P < 0.05$); however, milt volumes of the courting groups did not differ (Dunn's test, $P > 0.05$; Figure. 3.1A). Post test milt volumes exceeded pre-test volumes in 5 of 12 males in each of the courting groups. Although there was a tendency for greater serum LH concentrations in courting than in control groups, differences among groups were not significant (ANOVA, $F_{2,27} = 2.127$, $P = 0.139$; Figure 3.1B). However, when data from both courting groups were combined and compared to controls, the difference in serum LH concentrations was significant (unpaired t-test, $t = 2.071$, df = 28, $P = 0.048$).

3.3.2 Experiment 2: 90 Minute Courtship

Body weights did not differ among the three treatment groups (ANOVA, $F_{2,33}$ = 0.047, P = 0.954; Table 3.1). Prior to testing, milt volumes were comparable among groups (Kruskal-Wallis test, H = 0.155, P > 0.926, each group N = 12; Figure 3.2A). Post-test milt volumes were smaller than pre-test volumes in control males (Wilcoxon test, T_{12} = 64.000, P = 0.009), but not in small and large males (Wilcoxon test, small male, T_{12} = 36.000, P = 0.176, large male, T_{12} = -44.000, P = 0.092; Figure 3.2A). Although post test milt volumes did not differ among groups (Kruskal-Wallis test, H = 5.590, P = 0.061, each group N = 12), there was a trend for milt of males courting large females to be higher than that of males courting small females (e.g. post-test milt exceeded pre-test volumes in 8 of 12 males with large females, but only in 5 of 12 males placed with small females). Post-test concentrations of serum LH and T did not differ among groups (LH concentration, ANOVA, $F_{2,33}$ = 2.379, P = 0.108; T concentration, Kruskal-Wallis test, H = 4.496, P = 0.106, large and control males, N = 11, small males, N = 12; Figure 3.2A, B, C); however, when data from both courting groups were combined and compared to controls, the difference in serum concentrations of LH and T were significant (LH concentration, Welch's unpaired t-test, t = 2.676, df = 33, P = 0.012; T concentration, Mann-Whitney test, $U_{11,23}$ = 69.000, P = 0.034).

3.4 Discussion

Female body size did not affect courtship-induced milt or endocrine responses in males. Spawning behavior with a PGF-injected female was effective in stimulating milt production (Figure 3.1A, 3.2A) and elevating circulating LH (Figure 3.1B, 3.2B) and T concentrations (Figure 3.2C) in courting males after 30 or 90 minutes and these results are in accordance with previous findings (Stacey and Sorensen, 2002). However, males courting small females and those courting large females had equivalent volumes of strippable milt, LH and T.

As in males of many vertebrates including fish (Wingfield et al., 1990; Oliveira et al., 2002), socio-sexual interactions increase circulating LH and steroid hormones in male goldfish (Kyle et al., 1985; Dulka et al., 1987a; Sorensen et al., 1989). Surprisingly, the LH increase typically observed in spawning male goldfish does not mediate the increase in milt volume that also accompanies spawning, because the latency for spawning-induced milt increase is too short for an LH-mediated effect, and because spawning-induced milt increase also occurs in acutely hypophysectomized males (Zheng and Stacey, 1996). Although the function of spawning-induced endocrine responses in male goldfish is not known, it seemed reasonable to expect that, if these endocrine changes are a component of male competition, the magnitude of these changes might reflect differential stimuli from females.

Spawning with a PGF-injected female does not always increase serum LH. For example, Kyle et al. (1985) found that serum LH concentrations increased after 20 minutes of spawning with a PGF-injected female and remained elevated during the 60 minute test period, whereas Sorensen et al. (1989) found that spawning with a PGF-

injected female increased serum LH only in some of their reported experiments. Furthermore, time of day appears to influence the LH response, as exposure to PGF-injected females has been reported to increase serum LH concentration during the night but not during the day (Dulka et al., 1987b; Hontela and Stacey 1990).

This experiment did not investigate sperm allocation (sperm release) directly, but only measured milt volume as a proxy. Results may have been different if males had the opportunity to engage in spawning behavior, in addition to courting behavior. Although the volumes of milt stripped from the sperm ducts were comparable in males that courted large and small females, it is possible that males allocate more sperm to reproductive events with large females by increasing the number of sperm released. Indeed, studies conducted in a natural setting found that *T. bifasciatum* and *S. radians* males released more sperm when spawning with large females (Shapiro et al., 1994; Marconato and Shapiro, 1996). In goldfish, a mechanism controlling sperm release by the contractile motion of the sperm ducts has been identified (Dulka and Demski, 1986) and male goldfish could use this mechanism to modulate sperm release based on female body size.

As in common carp (*Cyprinus carpio*; Blazer, 2002), female goldfish have asynchronous oocyte development, in which oocytes vary in their developmental stage during the protracted periods of vitellogenesis. Therefore, it is likely that the female size/fecundity relationship in goldfish is weaker than in species with group synchronicity in oocyte development that ovulate and spawn at regular daily, lunar or annual intervals. A clear size/fecundity relationship is expected for the first ovulation of the spawning season, but not for subsequent ovulations, which appear to be of variable number and timing. If this is the case, there may have been little selection pressure for males to

allocate more sperm to spawns with bigger females. Additionally, male goldfish may have never faced the selection pressure for a graded milt response because other factors, like the presence of multiple competitors, may always create situations of high sperm competition. Thus, males might gain far more by allocating sperm in response to competition than they would by allocating in response to female size. Although males are capable of distinguishing between the sexes based on visual cues (Thompson et al., 2004), the results of this study demonstrate that the combined differences in visual, tactile and olfactory stimuli from large and small females did not influence the milt response in males and support a mechanism of milt increase mediated primarily by olfactory and sexual stimuli.

Studying sperm release might provide a very different perspective on sperm allocation than studying milt volume. I explored several techniques to quantify sperm release but was unsuccessful in finding a reliable method. The major problem was that high levels of particulate matter in the Aquatic Facility water made it difficult to identify sperm in tank water, given the enormous dilution of sperm in the tank and the lack of specific sperm markers. Filters were used to reduce particulate matter in the water and efforts were made to recover as much of the sperm sample as possible.

Because spawning results in low sperm numbers in aquarium water, I evaluated a variety of filtration and centrifugation techniques to concentrate large (up to 20 liter) samples of aquarium water. These techniques produced physical damage to the sperm cells and made it difficult to identify them with certainty. Animal cell stains such as methylene blue and Richardson's purple, were added to reduce the ambiguity. However, these are general stains that mark cells with different intensities and it is this staining

pattern that confers specificity. Because the head of a sperm cell is small (approximately 4 μm in diamter; personal observation), it was almost impossible to determine a staining pattern with a light microscope.

Flow cytometry had not been used previously to quantify sperm release, but a procedure using flow cytometry and a dual florescent dye kit seemed promising. The dyes SYBR 14 (Invitrogen, Eugene, OR, USA) and propidium iodide have been used to count live and dead sperm cells in milt samples from the common carp, Siberian sturgeon (*Acipenser baerii*), tench (*Tinca tinca*) and wels (*Silurus glanis*; Flajšhans et al., 2004). In addition, flow cytometry has been used to sort two populations of spermatozoa labeled with florescent dyes from bull (Thomas et al., 1998) and turkey (Donoghue et al., 1995) semen samples. The florescent dyes marked the sperm cells in a stripped milt sample from goldfish; however, the florescent signal degraded to levels indistinguishable from background when a sample of sperm added to a large volume of tank water was analyzed by the flow cytometer.

Beyond sperm release, the ultimate goal of sperm allocation studies in goldfish would be to determine whether males fertilize more eggs when spawning with large females. Female size and fertilization rate could be explored by giving a male the opportunity to spawn with two ovulated females of differing size and then incubating the eggs until hatching to determine maternal identity through microsatellite DNA fingerprinting. Previous studies validate microsatellite DNA fingerprinting as a reliable method of testing paternity in goldfish (Zheng et al., 1997).

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Table 3.1. Body weight (mean ± SEM; range) of males used in Experiment 1 and Experiment 2.

Experiment	Treatment		
	Control	Spawn with small female	Spawn with large female
Experiment 1 (30 min test)	58.5 ± 4.3 (29.4 – 75.1)	61.5 ± 4.2 (41.5 – 82.6)	58.6 ± 3.5 (45.1 – 78.3)
Experiment 2 (90 min test)	75.8 ± 4.8 (54.0 – 100.3)	76.2 ± 4.2 (51.4 – 100.3)	77.7 ± 4.3 (54.5 – 97.7)

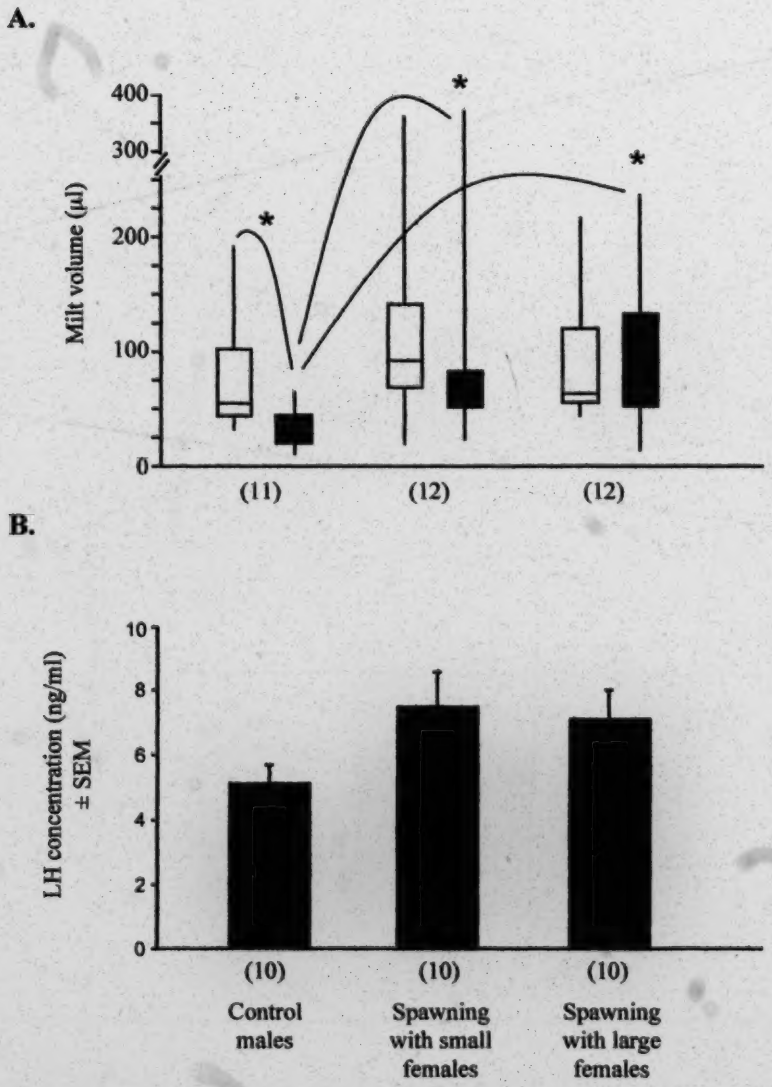

Figure 3.1. (A) Milt volume (median, quartile range and range) prior to (clear bars) and following (filled bars) 30 minutes of isolation (control) or 30 minutes of spawning with small females or spawning with large females and (B) serum LH (mean ± SEM) of the same three treatments. Sample sizes in parentheses. * = $P < 0.05$

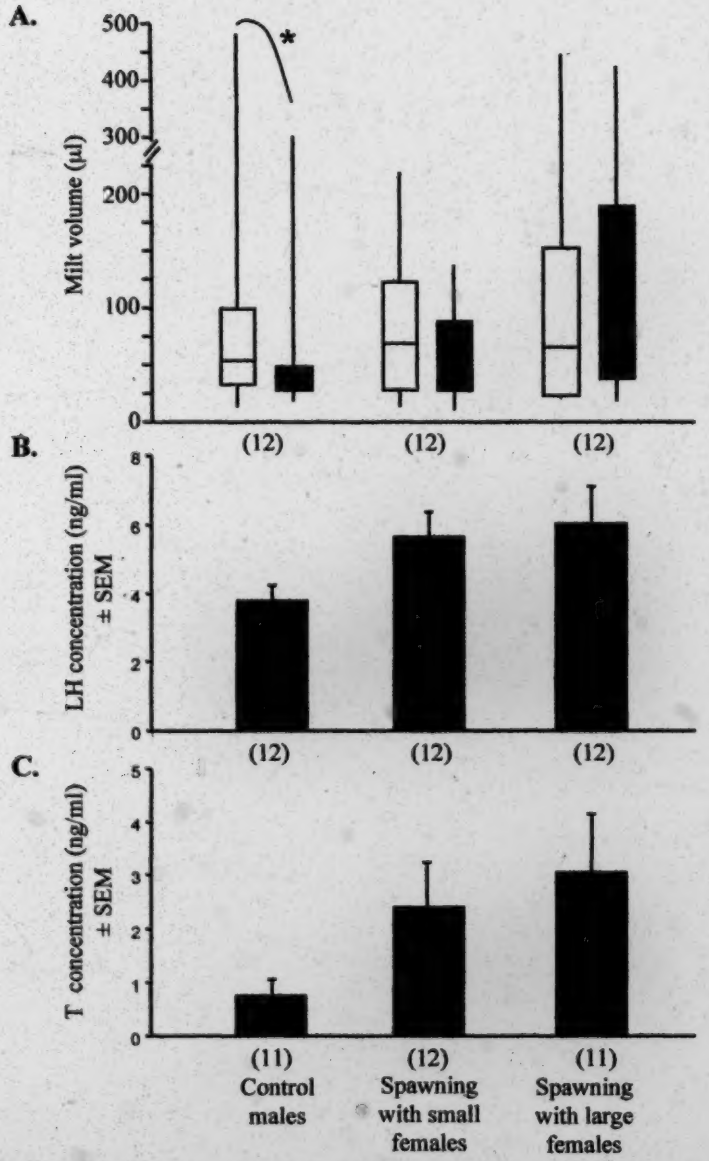

Figure 3.2. (A) Milt volume (median, quartile range and range) prior to (clear bars) and following (filled bars) 90 minutes of isolation (control) or 90 minutes of spawning with small females or spawning with large females, (B) serum LH (mean ± SEM) and (C) serum T (mean ± SEM) of the same three treatments. Sample sizes in parentheses. * = $P < 0.05$

4. EFFECT OF MALE COMPETITION ON MILT VOLUME AND BLOOD HORMONES

4.1 Introduction

In externally fertilizing fishes, the effect of competitors on the quantity of released sperm is predicted by theoretical models (Ball and Parker, 1997) that assume males can directly assess the number of competitors, modulate the number of sperm released, and manufacture and store sperm prior to ejaculation. If sperm competition proceeds by a *fair raffle* (Parker, 1990), then a male will gain the most benefit by releasing more sperm when there is a single competitor because he will increase his probability of fertilizing more of the eggs. As the number of competitors increases further, however, sperm release should decrease because the contribution of a single male will be insignificant when compared to the amount of sperm produced by the competitors (Ball and Parker, 1997).

Laboratory studies provide some empirical evidence that male fishes adjust sperm allocation in response to competitors. Prior to oviposition, male Amur bitterlings (*Rhodeus sericeus*) increased ejaculation rate when in the presence of a single competitor but decreased ejaculation rate when in the presence of 4 – 6 competitors (Candolin and Reynolds, 2002). Although sperm release was not measured directly, the size and density of the ejaculate cloud from the focal male did not differ between the density treatments (Candolin and Reynolds, 2002). Similarly, male rainbow darters (*Etheostoma caeruleum*) increased sperm release when exposed to chemical and visual cues from either 1 or 4 male competitors (Fuller, 1998). However, males did not modulate sperm release in response to the number of competitors (Fuller, 1998). Fuller (1998) suggests

that the guarding male may forgo spawning instead of modulating sperm release in the presence of 4 competitors, because the benefits to his reproductive success are drastically reduced and because he is more assured of other mating opportunities.

Male grass gobies (*Zosterisessor ophiocephalus*) and black gobies (*Gobius niger*) gain mating opportunities with females by guarding territories or sneaking matings. In an experiment where the number of sneaker males was manipulated, the focal sneaker male released most sperm when exposed to visual stimuli from a territorial mating pair and 1 other sneaker male (Pilastro et al., 2002). Contrary to the predictions of Ball and Parker (1997), this occurs in the presence of two other males. Interestingly, the number of sneaker males present does not influence the sperm released by territorial males (Scaggiante, et al., 2005). Instead, territorial males increase the frequency of patrolling and attacking behaviors (Scaggiante, et al., 2005). Further, in a natural setting, group-spawning bluehead wrasse (*Thalassoma bifasciatum*) released approximately 50 times more sperm than pair spawning wrasse (Shapiro et al., 1994).

The effect of male competitors on sperm allocation has been studied in species where males are territorial and use alternate mating strategies but has yet to be investigated in a non-territorial, promiscuous species like the goldfish. In the goldfish, females ovulate synchronously and neither sex provides parental care. During spawning, multiple males court a female and males are engaged in intense sperm competition. Males increase and decrease milt volume in a complex manner under various social conditions (Stacey and Sorensen, 2006). Many studies have shown that spawning interactions affect both milt volume and luteinizing hormone (LH) (Kyle et al., 1985; Sorensen et al., 1989); because these gonadal and endocrine responses are likely

components of male sperm competition (Stacey and Sorensen, 2006), it is reasonable to assume that the magnitude of these responses are affected by the presence of competitors. Based on the Ball and Parker model (1997), it is predicted that males will increase sperm allocation when in the presence of a single competitor. Experiments were conducted to determine if the presence of a male spawning competitor affects the milt or endocrine responses to sexually active females.

4.2 Methods

4.2.1 Experimental Animals

Goldfish of the comet variety were obtained annually from either Aquatic Imports (Calgary, Canada) or Huntington Creek (Maryland, USA). Shipments were made in the springtime between March and June, just prior to the reproductive season. Fish were housed at the University of Alberta Aquatics Facility in 300 L holding tanks with a continuous inflow of dechlorinated water. Holding tanks were maintained at a constant water temperature (17 °C) and photoperiod (16L:8D) and contained gravel and airstones. Fish were kept in same sex groups of approximately 40. Male goldfish were sexed based on the presence of tubercles on the operculum or on the pectoral fins. Fish were fed flake food and koi pellets *ad libitum*. Experiments followed the guidelines set out by the Canadian Council on Animal Care.

4.2.2 Experimental Protocol

The two experiments described below were conducted to determine whether the magnitudes of milt and endocrine responses to courtship were influenced by the presence

of male competitors. Male goldfish courted prostaglandin $F_{2\alpha}$ (PGF)-injected females for 15 minutes in experiment 1 and 90 minutes in experiment 2. As in chapter 3, it was important to investigate short and long time periods because males may vary in their milt response by altering the rate of filling or the duration of filling of the sperm ducts. Differences in the rate of filling should be apparent after 15 minutes of courtship because treatment males will not have time to fill sperm ducts to capacity. Alternatively, it is possible that males in competitive treatments may fill the sperm ducts at the same rate but for a longer duration than males in competitive treatments. Difference in the duration of filling should be evident after 90 minutes.

Experiment 1: 15 minute courtship

This experiment involves 3 male treatment groups, which were stripped of milt immediately prior to and following either a 15 minute period of courtship or a 15 minute period of isolation. Four to five days prior to experimental testing, 25 males of similar weight were placed in 60 L holding tanks in groups of five and 15 females (105.75g ± 3.68) were placed in 60 L holding tanks in groups of three. To ensure that males held similar milt volumes in the sperm ducts prior to treatment, males were stripped of milt immediately prior to testing as described by Stacey et al. (2001). Briefly, males were anaesthetized (2-phenoxyethanol; 0.05%, Syndel, Vancouver, Canada) and placed upside down in a moist, slotted foam pad. Gentle pressure was applied to the abdominal area, just anterior to the gonopore and milt was drawn into pre-weighed hematocrit tubes until milt could no longer be expressed. Males were revived and returned to their holding aquaria. To induce receptive behavior, females received an intra-muscular injection of

PGF at an approximate dose of 200 ng per g body weight and were placed individually in the testing aquaria. Testing aquaria (60 L) contained gravel and an air stone. Experiments were conducted in the absence of spawning substrate to minimize the chance that the male would release milt during the test period. From a single holding tank containing five males, two were transferred to a test tank containing a female (competitive treatment), two were transferred to separate tanks each containing a female (non-competitive treatment), and the fifth (control) male remained in the holding tank. The 15 minute test period for each non-competitive male began with his first display of chasing (courtship) behavior, and for each competitive pair began when the second male initiated courtship. After 15 minutes of courting, the four courting males and the control male were anaesthetized, stripped of milt a second time, bled from the caudal vasculature by syringe, and returned to their holding tanks. Controls were stripped after the competitive and non-competitive groups. The hematocrit tubes were weighed and the calculated milt sample weights were expressed as volumes, assuming a milt density of 1.0 g/ml.

Blood samples were allowed to clot on ice for 3-4 hours before being centrifuged. Serum was transferred to a new centrifuge tube with a preservative (1 µl thimerosol; 1%), frozen on dry ice and stored at –20 °C. Serum LH was determined by a carp radioimmunoassay (RIA) as described by Peter et al. (1984). Serum testosterone concentration (T) was not assessed because blood serum volume was limited.

Experiment 2: 90 minute courtship

This experiment was conducted in the same way as experiment 1, except that males ($N = 45$) were allowed to court for 90 minutes before milt and blood samples were

taken. Additionally, serum T was determined by an enzyme linked immunosorbent assay (ELISA) kit (MP Biomedicals; Orangebury, NY, USA) according to the manufacturer's instructions.

4.2.3 Statistics

In experiment 1, body weight data were analyzed using Kruskal Wallis test because attempts to transform these data to create data sets with Gaussian distributions did not work. However, body weight data were not transformed in experiment 2 and were analyzed using ANOVA. Milt volume differences among groups were tested using Kruskal Wallis test. Milt volume pre-test and post-test differences within groups were tested using Wilcoxon matched-pairs signed-rank tests (Instat V. 3, Graphpad Software, Inc., San Diego, CA, USA). Serum LH and serum T concentrations were transformed (log) and analyzed by ANOVA and Tukey-Kramer Multiple Comparisons tests (Instat V. 3, Graphpad Software, Inc., San Diego, CA, USA). All statistical tests were two-tailed and evaluated at the $\alpha = 0.05$ significance level.

4.3 Results

4.3.1 Experiment 1: 15 Minute Courtship

Male body weight did not differ among the three treatment groups (Kruskal-Wallis test, $H = 0.013$, $P = 0.994$, competitive and non-competitive male groups, $N = 10$, control group, $N = 5$; Table 4.1). Pre-test milt volumes were equivalent among the groups (Kruskal-Wallis test, $H = 0.101$, $P = 0.951$, competitive and non-competitive male groups, $N = 10$, control group, $N = 5$) as were post-test milt volumes (Kruskal-Wallis test,

$H = 1.712$, $P = 0.425$, competitive and non-competitive male groups, $N = 10$, control group, $N = 5$; Figure 4.1A). When data from both courting groups were combined and compared to controls, post-test milt volumes did not differ (Mann-Whitney test, $U_{5,20} = 35.000$, $P = 0.336$). Although there was a tendency for higher LH concentrations in courting males, serum LH was not different among groups (ANOVA, $F_{2,18} = 0.537$, $P = 0.593$; Figure 4.1B), even when all courting males were compared to controls (unpaired *t*-test, $t = 1.053$, df = 19, $P = 0.306$).

4.3.2 Experiment 2: 90 Minute Courtship

Male body weight did not differ among groups (ANOVA, $F_{2,42} = 0.374$, $P = 0.690$, Table 4.1). Post-test milt volumes were lower than pre-test volumes in control males (Wilcoxon test, $T_9 = 45.000$, $P = 0.004$) but not in non-competitive and competitive males (Wilcoxon test, non-competitive males, $T_{18} = 73.000$, $P = 0.119$, competitive males, $T_{18} = -1.000$, $P > 0.999$; Figure 4.2A). Pre-test milt volumes were not different among groups (Kruskal-Wallis test, $H = 0.189$, $P = 0.910$, competitive and non-competitive male groups, $N = 18$, control group, $N = 9$; Figure 4.2A). Although post-test milt volumes of courting males were greater than those of controls, only the difference between competitive and control males was significant (Kruskal-Wallis test, $H = 7.495$, $P = 0.024$, competitive and non-competitive male groups, $N = 18$, control group, $N = 9$; Dunn's test, $P < 0.05$; Figure 4.2A). Serum LH and T concentrations were higher in the courting groups (LH concentration, ANOVA, $F_{2,39} = 5.583$, $P = 0.007$; Tukey-Kramer test, non-competitive male vs. control $P < 0.01$, competitive male vs. control male $P < 0.05$; T concentration, ANOVA, $F_{2,30} = 10.663$, $P = 0.0003$; Tukey-Kramer

test, non-competitive male vs. control $P < 0.001$, competitive vs. control $P < 0.001$); however, hormone concentrations of non-competitive and competitive males did not differ (Tukey-Kramer, $P > 0.05$; Figure 4.2B, C). Serum LH concentrations and serum T concentration were strongly correlated (linear correlation; $r = 0.709$, $P < 0.0001$, $N = 30$).

4.4 Discussion

These experiments were the first to investigate the effect of competitors on sperm allocation in a non-guarding, promiscuous fish. In contrast to the predictions made by Ball and Parker (1997), the presence of male competitors did not influence sperm allocation in male goldfish. Post-test milt volume did not differ between courting groups, indicating an absence of a competition effect after 15 or 90 minutes of courtship (Figure 4.1A, 4.2A). Similarly, endocrine response was not affected by the presence of competitors. Serum LH concentrations did not differ among groups after 15 minutes (Figure 4.1B) and, although LH and T were elevated in the courting groups after 90 minutes (Figure 4.2 B, C), there was no difference between courting groups.

Again, this experiment did not investigate sperm allocation (sperm release) directly, but only measured milt volume as a proxy. Results may have been different if males had the opportunity to engage in spawning behavior, in addition to courting behavior. It is possible that males allocate more sperm when in the presence of a single competitor by increasing the number of sperm released. However, a reliable method to measure sperm release has yet to be developed.

Possibly, the presence of competitors did not affect milt response because male goldfish may always experience intense competition for mating opportunities. As a

result, there may be no need for a finely tuned milt response based on the number of competitors. Firstly, the number of ovulated females is limited. Although water temperature and photoperiod influence the timing of ovulation, females can ovulate multiple times and ovulation is not precisely synchronous (Kobayashi et al., 1988). Secondly, a pheromone cue can stimulate numerous males directly and indirectly. For example, direct exposure to 17,20βP at picomolar concentrations can stimulate milt increase in males (Dulka et al., 1987) which in turn can stimulate milt production in other males (Fraser and Stacey, 2002). Consequently, there is the potential in many instances for the number of stimulated males to be much greater than the number of ovulated females and for competition to be intense.

There is some evidence to suggest that males can use visual cues to distinguish between males and females during the breeding season (Thompson et al., 2004). However, in a natural setting, ovulation occurs in late scotophase (Stacey et al., 1979) and it is likely that spawning normally begins in very low light levels. Also, spawning probably takes place in turbid water where visual cues are minimal. Thus, males may not be able to accurately assess the number of competitors in the area. Under these conditions, it would be beneficial for males to produce milt without regard to the precise number of competitors.

In species such as the black and grass goby, territorial males respond to the presence of sneaker males by increasing guarding and attacking behaviors instead of sperm release (Scaggiante et al., 2005). Although goldfish do not guard territories, resources or mates, males may become more aggressive during spawning. Androstenedione (AD) is a steroid released by females as one of three components

comprising the preovulatory pheromone and released by males during reproduction. In behavioral tests, males exposed to nanomolar concentrations of AD increased pushing behavior (Poling et al., 2001). Males may use increased aggression and physical force to dominate other males or secure a closer position to the female. Furthermore, males may alter their behavior by choosing not to spawn when there are large numbers of competitors. Male darters were more likely to forego spawning opportunities when there were four competing males present (Fuller, 1998). If male goldfish alter their behavior in the presence of competitors, it may not be necessary to modulate their sperm response.

Further investigations should concentrate on sperm release and fertilization rate, because the volume of strippable milt may not indicate the amount of sperm released in the presence of competitors. In goldfish, sperm release is controlled by contraction of the sperm ducts (Dulka and Demski, 1986). Males may use this mechanism to modulate sperm release when competitors are present. As mentioned previously, a reliable method for quantifying sperm release needs to be developed for goldfish.

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Table 4.1. Body weight (mean ± SEM; range) of males used in Experiment 1 and Experiment 2.

Experiment	Treatment		
	Control	Non-competitive spawning	Competitive spawning
Experiment 1 (15 min test)	55.6 ± 7.7 (28.2 – 73.2)	56.3 ± 5.1 (28.8 – 80.6)	54.9 ± 6.0 (35.9 – 79.4)
Experiment 2 (90 min test)	57.4 ± 4.6 (23.7 – 80.3)	52.3 ± 4.1 (35.0 – 99.1)	51.9 ± 3.9 (34.9 – 85.9)

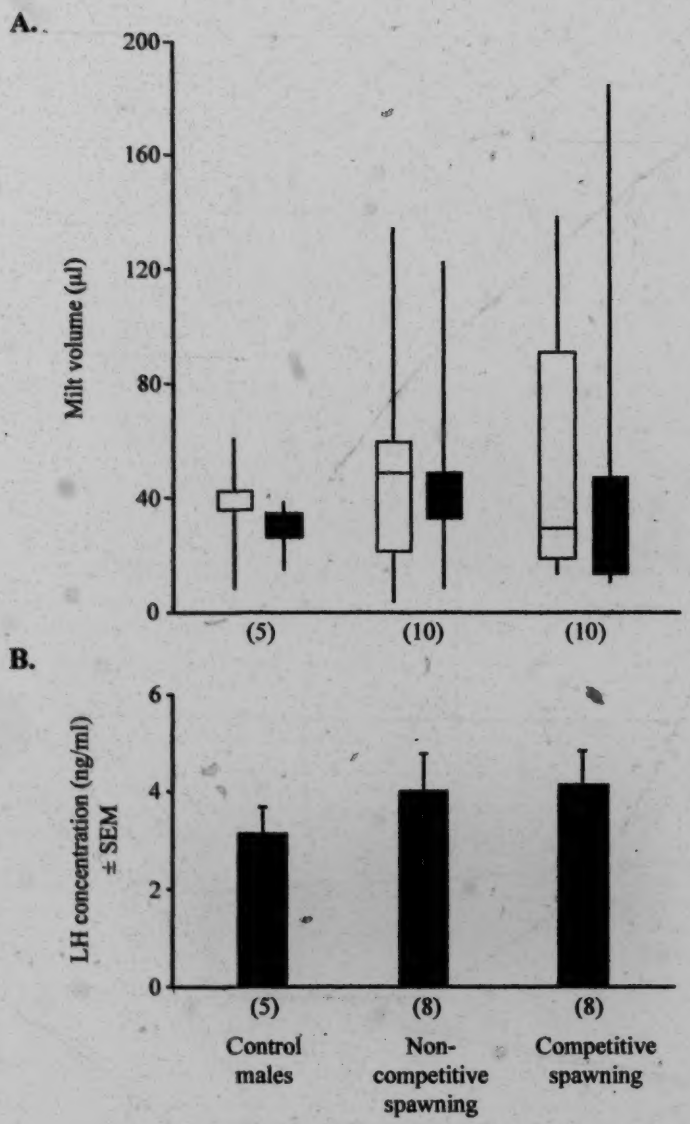

Figure 4.1. (A) Milt volume (median, quartile range and range) prior to (clear bars) and following (filled bars) 15 minutes of isolation (control) or 15 minutes of non-competitive or competitive spawning, (B) serum LH (mean ± SEM) of the same three treatments. Sample sizes in parentheses.

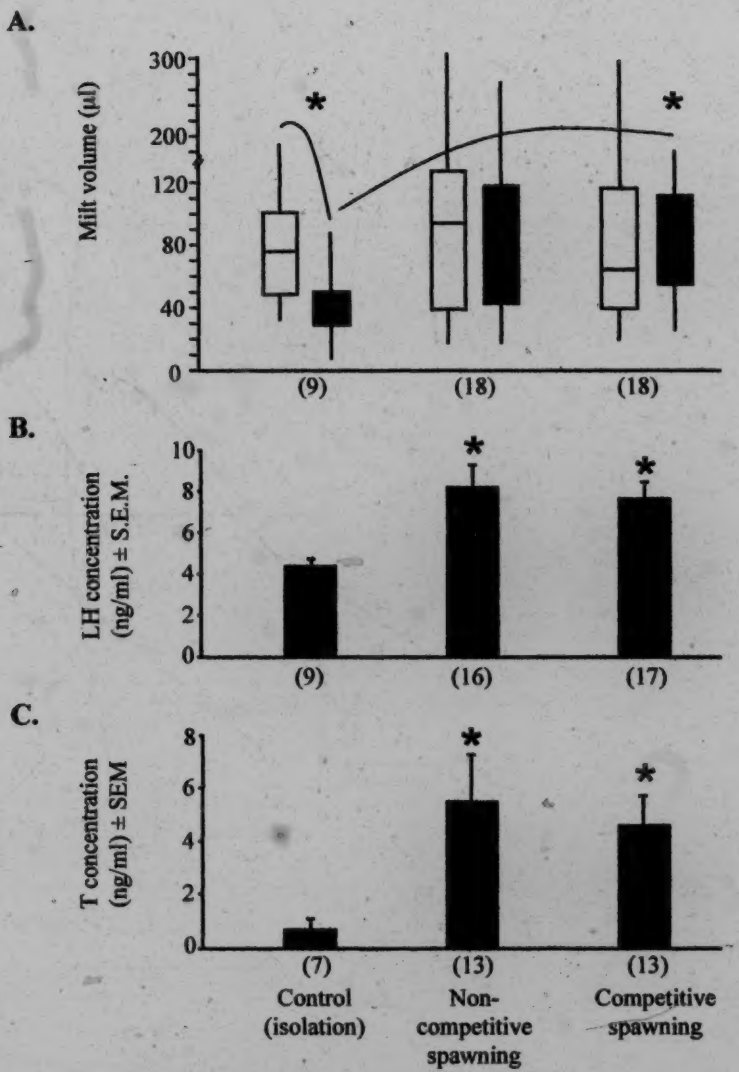

Figure 4.2. (A) Milt volume (median, quartile range and range) prior to (clear bars) and following (filled bars) 90 minutes of isolation (control) or 90 minutes of non-competitive or competitive spawning, (B) serum LH (mean ± SEM), (C) serum T (mean ± SEM) of the same three treatments. Sample sizes in parentheses. * = $P < 0.05$

5. CONCLUSIONS

This research investigated the effect of female body size and the presence of competitors on mate choice, sperm allocation and endocrine response in the male goldfish (*Carassius auratus*), a promiscuous fish species in which males engage in intense sperm competition. Male mate choice was dependent on relative body size in that male goldfish discriminated against females smaller than themselves. Despite this behavioral response to female size, I found no evidence that males adjusted milt volume to female size, although, as in previous studies (Kobayashi et al., 2002), males increased milt volume, LH, and T during courtship. Similarly, I found no evidence that male goldfish adjust the magnitude of their courtship-induced milt and endocrine responses in the presence of competitors.

Although these studies have given some insight into mate choice and sperm allocation in male goldfish, many important questions remain. For example, the effect of female body size and competitors on sperm release need to be examined because sperm release may not be closely related to milt volume. Female size (Shapiro et al., 1994; Marconato and Shapiro, 1996) and competitors (Candolin and Reynolds, 2002) have been shown to influence the number of sperm released in some fishes and it is quite possible that goldfish may also allocate sperm release (Dulka and Demski, 1986). Additionally, it is unknown how competition might affect mate choice. In the absence of competition, male goldfish prefer large females but such situations are probably rare in nature given that males are promiscuous and engage in intense sperm competition. Further investigations should focus on determining how the pattern of mate choice observed in the absence of competition is affected by the number and size of competitors.

In conclusion, because of the large amount of information on the effects of female pheromones and male stimuli on milt production (Stacey and Sorensen, 2002; 2006), the goldfish offers the best available model for looking at these phenomena in a promiscuous non-guarder which may be typical of many other cyprinids with similar mating systems.

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